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CHEMISTRY OF THE PROTEINS

AND

ITS ECONOMIC APPLICATIONS

By

DOROTHY JORDAN LLOYD

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R h A ti n

Introduction by

SIR FREDERICK GOWLAND HOPKINS

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WITH 50 ILLUSTRATIONS

LONDON

J & A CHURCHILL

7 GREAT MARLBOROUGH STREET

1926

TO

J L AND M A J L

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INTRODUCTION

ALTHOUGH English books dealing with the chemistry of proteins are not lacking the present work stands alone in aim and in scope It contains a synthesis of knowledge such as has not hitherto been attempted in this domain The author has explored every branch of literature which could possibly bear upon the subject she has assembled facts won in diverse lines of study and yet secures unity and continuity in her presentation of the whole

The subject falls of course naturally into two main divisions the constitutional chemistry of proteins and their physical chemistry In a formal sense these are dealt with separately in this book but the bearing of one upon the other is kept clearly in view and the somewhat unhappy divorce which these two aspects of chemical knowledge are apt to suffer is not found here Few I think could have dealt with both aspects with the grasp and mastery of essentials which the author displays

While treating with completeness from a chemical standpoint each of these broad divisions of the subject the book contains references to every important application of the facts Their bearing upon general biochemistry and upon physiology and pathology is consistently indicated throughout and even their commercial applications receive due notice Although so exhaustive the work is no mere compilation It is written from a standpoint which is fully critical though it does not exclude reference to views which while not yet proven are suggestive and stimulating

Even the specialist may profit from the author s synthetic labours Even he is apt at times to keep different branches

of his knowledge too far apart in his mind He may well find as the result of reading this book that the fertile mating of facts which for him were previously disconnected has yielded him a progeny of happy suggestion for research Advanced students of physiology and biochemistry may feel confident that if they possess this book they can easily acquaint themselves with all that is significant in our present knowledge of proteins while more junior students even if they do not need to know all that the author has to tell will find that certain of the fundamental aspects of the subject especially on the physico chemical side which may have proved difficult when read elsewhere are discussed with a clarity that they will appreciate Those whose interests are more purely chemical will discover here an assemblage of highly significant facts which because of their publication in biological journals may easily have escaped their notice

I am so assured of the merits of Dr Jordan Lloyd's work that I take great pride in being allowed to write this foreword

F GOWLAND HOPKINS

PREFACE

In the autumn term of 1924 the author was asked to give a course of lectures at the Battersea Polytechnic on The Chemistry of the Proteins and its Industrial Applications The present book has developed from the notes originally put together as the basis of the lectures the primary object of which was to give a general review of protein chemistry

At present there is no easily accessible record in the English language of the position of protein chemistry as it has been affected by the development of the last few years This volume is intended to remedy the defect by providing a concise account of the theories of protein chemistry and of their practical applications to biology and industry

The first part of the book deals with the fundamental units in the protein molecule methods of protein analysis the different classes of proteins the identification of individual proteins the structure of protein molecules the types of linkage found and their reactions to the proteolytic enzymes A chapter is included embodying all the recent work on the position of the proteins in the important science of dietetics and another on the problems and methods of food preservation

The second part of the book deals with the physical chemistry of the proteins the nature of the combination between proteins and acids or alkalis the properties of protein sols and gels osmotic pressure viscosity swelling etc and the applications of the colloidal chemistry of the proteins to problems of physiological interest The evidence on the influence of neutral salts on protein solutions is summarised and a chapter on the theory of protein solutions has been included Other chapters deal with the formation and properties of gels the clotting of blood and milk the denaturation and coagulation of proteins the drying of

proteins and the uses of proteins in industry The bio chemistry of the manufacture of wool silk leather adhesives glazes sizes and emulsions is dealt with in outline

The writer has also endeavoured to gather together a comprehensive bibliography of the whole subject The literature is so vast that completeness is impossible and many interesting and important papers have necessarily been omitted It is hoped that the references given are sufficiently numerous and varied to provide clues for the discovery of these

In completing a work of this scope it is a pleasant duty to acknowledge the assistance of many friends In the first place the writer wishes to express her thanks to those editors of journals who have kindly allowed her to illustrate her text by means of figures originally published under their auspices To the authors of these papers her sense of indebtedness is also gratefully acknowledged Her cordial thanks are also due to Dr R H Pickard F R S Principal of the Battersea Polytechnic and Director of the British Leather Manufacturers Research Association for his sympathetic interest in the projected work and also for invaluable criticism at the proof stage To Dr R Robison Miss M Stephenson Mr H Raistrick and Mr A E Caunce who have read the manuscript and offered valuable suggestions her thanks are also offered

The writer also wishes to express her gratitude to Professor Sir Frederick Gowland Hopkins M D F R S for writing the Introduction to this book but still more as all those who have had the privilege of working in his laboratory will understand for the continual inspiration that comes from contact with his genius and for the kindly encouragement that his generosity metes out to all his pupils awakening in many of them faculties which might otherwise have remained dormant

DOROTHY JORDAN LLOYD

London

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CHEMISTRY OF THE PROTEINS

PART I

CHAPTER I

THE NATURE AND CONSTITUTION OF THE PROTEINS

Occurrence—Composition and Characteristics—The Colour Tests for Proteins—Precipitation Tests—Constitution—Units of the Protein Molecule the Amino Acids the Non amino Units—Fischer s Method of Protein Analysis—Synthesis of the Poly peptides—The Nature of the Protein Molecule

Occurrence

THE proteins are the chemical foundations of all living matter They form with water the material out of which are built the protoplasm of both animal and vegetable cells If a living cell be compared to a machine then the proteins are to be regarded as the material out of which the engine is built Their presence is intimately connected with the cycles of chemical and physico chemical change that constitute the life of the cell but under normal conditions in the adult organism they are not themselves oxidised to release the energy required to perform the work of the cell The fuel consumed for this end is supplied by other materials—carbohydrates fats etc Truly in times of fuel shortage the living machine has at its command a resource denied to an ordinary engine for it can supply emergency fuel by oxidising its own substance *i e* the cell can and does in periods of starvation feed on its own proteins In some

invertebrates this process can go on to such an extent that not only cell substance but even tissues *e g* the organs of reproduction can be absorbed in times of starvation and regenerated in times of plenty (see Jordan Lloyd 1914) Proteins therefore form the substance of the living body of all plants and animals and exist in every cell They are not accumulated in storage reservoirs or reserve food supplies except under conditions connected with the development of the young organism The series of rapid structural changes which follow the fertilisation of the ovum and end with completion of the adult form are such as necessitate a generous supply both of constructive material and of fuel It is therefore not surprising to find that the proteins besides forming the substance of the body in all plants and animals are also accumulated in eggs in seeds and in secretions intended as food for the growing young such as milk honey etc In addition to these sources animals as a biological group use proteins as a structural material out of which to fashion a number of accessory tissues—hair horn wool and silk are all proteins although they do not actually form a part of the substance of living protoplasm

Composition and Characteristics

The proteins consist of carbon (50 to 55 per cent) oxygen (25 to 30 per cent) nitrogen (15 to 19 per cent) hydrogen (7 per cent) frequently sulphur (0 5 to 2 5 per cent) and sometimes phosphorus They are characterised analytically by a group of colour reactions and by a series of precipitation tests The former are not specific for proteins as such but for certain groups commonly occurring in them The precipitation tests are typically those of amphoteric colloids

A few types of the proteins are insoluble in water The majority form colloidal solutions in water and for this reason cannot dialyse away through cell walls or membranes Only a few have ever been obtained crystalline These properties

suggest a large cumbersome molecule and the molecular weights of the proteins have been estimated variously at between 1 000 and 100 000 The protein molecule (or molecular complex) is formed by the condensation of a large number of molecules of various amino acids An amino acid is as its name suggests an organic acid containing an amino group Its general formula may be given as

$$R\ CHNH_2\ COOH$$

The presence of the carboxylic group gives it the properties of an acid the presence of the amino group those of a base It can therefore combine with either bases or acids in the first case forming the anion of the resulting salt in the second case the cation The simultaneous presence of carboxylic and amino groups gives the protein molecule its amphoteric character the very large number of amino acids condensed its colloidal character The different chemical groupings that can be substituted for R are responsible for the different colour reactions A remarkable fact in the constitution of proteins is that nearly all known amino acids are found in nearly all known proteins The differences between one protein and another are therefore due not so much to the nature of the component radicles as to the proportions in which these occur their arrangement and the manner in which they are linked

The Colour Tests for Proteins

(1) **The Biuret Reaction** (*Piotrowski*) —Proteins in a strong alkaline solution (sodium hydroxide) give a purple or pink colour on the addition of a few drops of dilute copper sulphate solution This reaction is due to the presence of two —CONH— groups attached to one another It is given by all proteins and their hydrolytic derivatives except the free amino acids and also by synthetic polypeptides prepared by the condensation of two or more amino acids

(2) **The Xanthoproteic Reaction** —Protein solutions give a

white precipitate with strong nitric acid On boiling this turns yellow and on the addition of strong sodium hydroxide orange This reaction is due to the presence of a benzene nucleus and indicates that the protein on hydrolysis yields tyrosine tryptophane or phenylalanine

(3) **Millon s Reaction** —Millon s reagent consists of a mixture of mercurous and mercuric nitrates dissolved in nitrous and nitric acids With protein solutions it gives a white precipitate which turns brick red on heating This reaction is due to the presence of a hydroxy benzene nucleus and indicates that the protein contains the tyrosine radical

(4) **The Glyoxylic Reaction** (*Hopkins and Cole Adamkiewitz*) —Protein solutions containing glyoxylic acid (prepared by reducing oxalic acid) if brought into contact with concentrated sulphuric acid give a purple colour at the junction of the two liquids This reaction is due to the presence of the tryptophane grouping

(5) **Voisenet s Reaction** (1905) —Protein solutions containing a trace of formaldehyde give a violet colour on the addition of saturated hydrochloric acid containing a trace of nitrous acid This reaction is due to the presence of the tryptophane grouping

(6) **Phosphoric Acid Reaction** —Romieu (1925) finds that cell proteins give a rose pink colour with syrupy phosphoric acid after gentle warming The colour is ascribed to the tryptophane radical The test is recommended for histological work

(7) **The Sulphur Reaction** —Many proteins after boiling with strong sodium hydroxide give a black precipitate on the addition of lead acetate This reaction indicates that the protein contains the cystine grouping Sulphur may also be present in proteins in other types of combination but does not then give the sulphur reaction

(8) *α* **Naphthol Reaction** (*Molisch*) —Protein solutions containing a trace of *α* naphthol if brought into contact with concentrated sulphuric acid form a reddish violet ring at the

junction of the two liquids This reaction is due to the presence of a carbohydrate grouping generally glucosamine

Two other colour reactions have been applied to pro eins These are Jaffe s and von Bitto s tests for carbonyl or ketonic groups A positive reaction is believed to indicate anhydride rings in the protein

(9) **Picric Acid Reaction**—A reddish brown colour is developed by some protein solutions on heating with picric acid and sodium hydroxide

(10) **The *m* dinitrobenzene Reaction**—A pink colour is developed by some proteins after heating with sodium hydroxide on the addition of *m* dinitrobenzene

Precipitation Tests

(1) In weakly alkaline solutions the proteins are precipi tated by salts of the heavy metals such as lead acetate mercuric or ferric chlorides and the sulphates of copper or zinc In weakly acid solutions they are precipitated by acids or salts containing a complex anion such as phosphotungstic acid phosphomolybdic acid tannic acid picric acid etc These two types of precipitation are almost certainly due to the agglutination of the charged colloidal particles by a polyvalent ion of the opposite sign and also probably in part to salt formation Under appropriate conditions the precipitation can be reversed

(2) Proteins can be precipitated from solution by strong solutions of salts saturated magnesium sulphate or saturated ammonium sulphate being generally used This reaction is really a dehydration and is reversible It is similar to the salting out of soaps

(3) Alcohol in 80 per cent concentration precipitates the proteins from solution This reaction is also a dehydration At low temperatures 0 C on the addition of water the precipitate redissolves but with many proteins at ordinary temperatures the precipitation is accompanied by chemical change and is irreversible Acetone has a similar action on protein solutions

Constitution

The protein molecule is a large and complex structure built up from a small number of different elements sometimes as few as four—C H O and N An elementary analysis obtained by a combustion gives therefore very little insight as to its constitution For this reason until methods had been devised for separating the molecule not into its component atoms but into its component groups or units little progress was made in the knowledge of its constitution The most important method of group analysis is hydrolysis in aqueous solution This can be effected in several ways (1) by acids (2) by alkalis or (3) by the proteolytic ferments pepsin trypsin etc The method most generally used is acid hydrolysis

It is obvious that the mere hydrolysis of a protein does not of itself give much information as to the original constitution From the mass of mixed hydrolytic products the various types of unit have to be separated recognised and quantitatively determined Since all the units are chemically very closely allied their separation has been and still is a matter of very great difficulty and the history of the discovery of the constitution of the proteins is mainly a history of the discovery of suitable methods of isolating the different units from the mixed mass of hydrolytic products Drechsel s method of hydrolysis with strong acid and separation of the hydrolytic products into a basic and a non basic fraction by precipitation with phosphotungstic acid is the one on which all later work has been founded From the basic precipitate lysine (Drechsel 1889) arginine (Hedin 1895) and histidine (Kossel 1896) were soon isolated The main bulk of the hydrolysed protein is however in the non basic filtrate and although units from this had been isolated no substantial progress was made in dealing with this fraction until 1901 when Emil Fischer separated the units by freeing them

from water converting them into esters and fractionally distilling under reduced pressure at a low temperature The separated esters were saponified and the free amino acids obtained finally in the crystalline state Fischer s method of separation of the non basic fraction remained predominant in protein chemistry until Dakin (1918) showed that an improved yield and separation of the component acids can be obtained by extracting the watery solution of hydrolytic products with butyl alcohol before proceeding to a final separation by esterification It is impossible here to give a list of the many important papers which give the results obtained by the use of Fischer s and Dakin s methods A very full bibliography of all work down to 1917 is to be found in Plimmer s valuable monograph on The Constitution of the Protein Molecule The more recent literature is given in Oppenheimer s Handbuch der Biochemie Bd I (1924)

The results of many analyses of hydrolysed proteins show that most proteins are built up almost if not entirely by condensation of amino acids or closely allied substances In no instance yet has the knowledge of the composition of any one protein been completed and the evidence of constitution now available has been slowly and laboriously collected from many sources The nature of the units found in the protein molecule may now be considered

Units of the Protein Molecule

On their first isolation from the products of protein hydrolysis some of the units proved to be substances already known others had previously been unknown For the method of isolation and the properties of the individuals the reader is referred to Plimmer s monograph or Oppenheimer s text book The majority of the units are amino acids which are always α amino acids that is one amino group is attached to the carbon atom next to the carboxylic group These groupings with the obvious exception of glycine pre exist in proteins in the optically active form

The amino acids may be conveniently classified according to the number of basic and acidic groupings which they contain This number in the case of either grouping may be one or two Hence the amino acids are usually classified as—

(1) Mono amino mono carboxylic acids
(2) Di amino mono carboxylic acids (basic)
(3) Mono amino di carboxylic acids (acidic)

The amino acids are sometimes classified on the constitution of the central nucleus which may be a fatty chain (straight or branched) a phenyl or substituted phenyl grouping or a heterocyclic ring

Besides the true amino acids certain allied groupings e g imino acids diketopiperazines ammonia etc have been isolated from hydrolysed proteins and form the non amino units of the protein molecule

THE AMINO ACIDS

(1) **Mono amino Mono carboxylic Acids**—*Glycine* glycocoll or amino acetic acid

$$CH_2NH_2\ COOH$$

occurs in large amounts in the hydrolysis products of gelatin and collagen

d Alanine or *d* α amino propionic acid

$$CH_3\ CHNH_2\ COOH$$

occurs in small amounts in the hydrolysis products of nearly all proteins and was isolated from silk by Weyl (1888)

l Serine or *l* β hydroxy α amino propionic acid

$$CH_2OH\ CHNH_2\ COOH$$

occurs especially abundantly in the hydrolysis products of silk from which it was first isolated by Cramer (1865)

d Valine *d* β dimethyl α amino propionic acid

$$(CH_3)_2CH\ CHNH_2\ COOH$$

is obtained in small amounts from many proteins

d Isoleucine *d* β methylethyl α amino propionic acid

$$(CH_3)(C_2H_5)CH \quad CHNH_2 \quad COOH$$

is obtained in small amounts from many proteins

d α *amino butyric acid*

$$CH_3 \quad CH_2 \quad CHNH_2 \quad COOH$$

Foreman (1913) reported the isolation of this acid from casein but its existence has been disputed

l Leucine *l* β dimethyl α amino butyric acid

$$(CH_3)_2CH \quad CH_2 \quad CHNH \quad COOH$$

obtained from many proteins and was isolated from horn by Hinterberger (1849)

d Norleucine *d* α amino caproic acid

$$CH_3 \quad CH_2 \quad CH_2 \quad CH_2 \quad CHNH_2 \quad COOH$$

has been found by Abderhalden and Weil (1913) as a unit in the protein of nerve tissue

l Phenylalanine *l* β phenyl α amino propionic acid

$$C_6H_5 \quad CH_2 \quad CHNH_2 \quad COOH$$

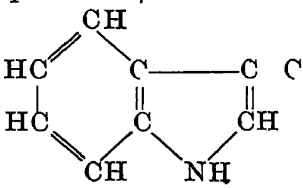
is obtained from many proteins and was isolated from squash seed globulin by Schultze and Barbieri (1881) It gives the xanthoproteic reaction

l Tyrosine *l p* hydroxy phenylalanine

$$C_6H_4OH \quad CH_2 \quad CHNH \quad COOH$$

occurs abundantly in the hydrolytic products of silk and in large amount from those of other proteins of an epidermal origin It was first isolated by Liebig (1846) from casein and by Hinterberger (1849) from horn It gives the xanthoproteic and the Millon reactions

l Tryptophane *l* β indole α amino propionic acid



is obtained in small quantities from most cell proteins It is

destroyed by acid hydrolysis and was first isolated by Hopkins and Cole in 1901 from a tryptic digest of casein It is responsible for the glyoxylic reaction

(2) The Basic Amino Acids (Di amino Mono carboxylic Acids) —*Lysine* ε α di amino caproic acid

$$CH_2NH\ CH_2\ CH_2\ CH_2\ CHNH_2\ COOH$$

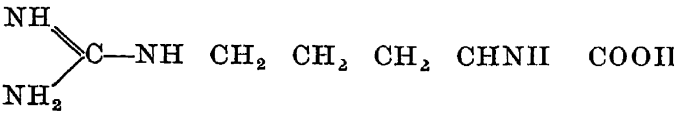
occurs fairly abundantly in the hydrolysis products of many proteins and was first isolated by Drechsel (1889) from casein

Hydroxylysine ε α amino β hydroxy caproic acid

$$CH_2NH_2\ CH_2\ CH_2\ CHOH\ CHNH_2\ COOH$$

was recently isolated by Schryver Buston and Mulherjee (1925) from isinglass in an optically inactive form

d Arginine *d* δ guanidine α amino valeric acid

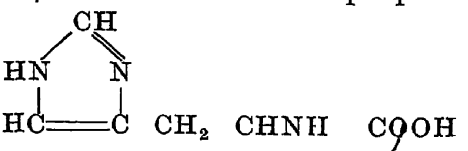


is obtained in quantity from ripe animal sperm and in large amounts from many vegetable seed proteins but was first isolated by Hedin (1895) from horn It is unstable in alkaline solutions being converted into ammonia and

ornithine δ α di amino butyric acid

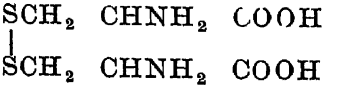
$$CH_2NH_2\ CH_2\ CH_2\ CHNH_2\ COOH$$

l Histidine *l* β imidazole α amino propionic acid



obtained in large amounts from the globin of red blood corpuscles was first isolated by Kossel (1896) from fish roe

Cystine di β thio α amino propionic acid



is obtained in large amounts from hair It was isolated

from horn serum albumin etc by Moerner (1898) It is destroyed in alkaline solutions and largely converted during acid hydrolysis into its reduced form —

Cysteine *β* thio *α* amino propionic acid

$$SHCH_2\ CHNH_2\ COOH$$

isolated by Embden (1901) from hair albumin etc Cystine and cysteine are lævo rotatory in acid solution

Neither histidine cystine nor cysteine are from their formulæ di amino mono carboxylic acids but their well marked basic properties and precipitation with phospho tungstic acid justify their inclusion in this group

(3) **The Acidic Amino Acids (Mono amino Dicarboxylic Acids)** —*l Aspartic acid* *l α* amino succinic acid

$$COOH\ CH_2\ CHNH_2\ COOH$$

was isolated by Kreusler (1869) from horn and is obtained from nearly all proteins In the vegetable proteins it is probably present as the acid amide *asparagine*

$$COOH\ CH_2\ CHNH_2\ CONH_2$$

which has been isolated in the free condition from growing seedlings

d Glutaminic acid *d α* amino glutaric acid

$$COOH\ CH_2\ CH_2\ CHNH_2\ COOH$$

was isolated by Horbaczewski (1879) from horn The free acid amide *glutamine*

$$NH_2CO\ CH_2\ CH\ CHNH_2\ COOH$$

has also been isolated from growing seedlings and in the vegetable proteins this unit is probably mainly present as the acid amide

β Hydroxy glutaminic acid

$$COOH\ CH_2\ CHOH\ CHNH_2\ COOH$$

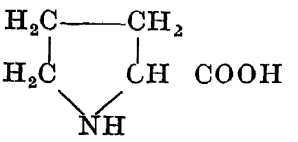
has been isolated by Dakin (1918) from casein It has very little optical activity in water but is dextro rotatory in hydrochloric acid solution

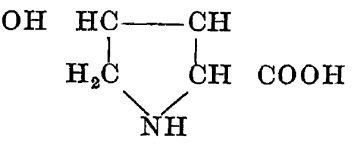
It is unlikely that many new amino acids of a form hitherto unrecognised remain undiscovered The trend of recent research does suggest however that the proteins also contain many of the known amino acid groupings in the form of hydroxyl derivatives Buston and Schryver (1925) believe they have isolated oxyaminobutyric acid and oxyvaline

A new sulphur containing acid of unknown constitution in which the linkages of the sulphur atom differ from those of cystine and cysteine has been described by Mueller (1921 1923)

THE NON AMINO UNITS

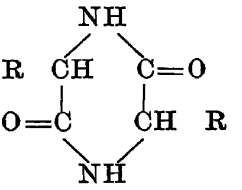
(1) The Imino acids

Proline  and

Hydroxyproline 

are obtained in large amounts from gelatin and abundantly from many of the proteins of cereal seed The former was isolated by Fischer (1901) from casein the latter from gelatin (1902) There has been some doubt as to whether they are really units in the protein molecule Knaggs and Schryver (1924) have shown that by repeated flocculation in an electric field the non amino nitrogen of gelatin may rise from 10 to nearly 20 per cent of the total nitrogen from which they conclude that proline is largely formed as a result of the treatment Sørensen and Anderson (1906) believe proline to be formed during hydrolysis by condensation of δ hydroxy α amino valeric acid Fischer and Bohner (1910) however and Abderhalden and Kautzsch (1912) consider that proline and hydroxyproline pre exist in the protein molecule

(2) **Diketopiperazines** the anhydrides of the dipeptides



condensation bodies which have a ring structure derived from condensing two amino acids have frequently been isolated from the syrupy residues of protein hydrolysis Abderhalden and Komm (1924) have brought forward strong evidence that these groupings pre exist in the protein molecule

(3) **Ammonia** is always released during protein hydrolysis apparently from the acid amide groups

(4) **Glucosamine**

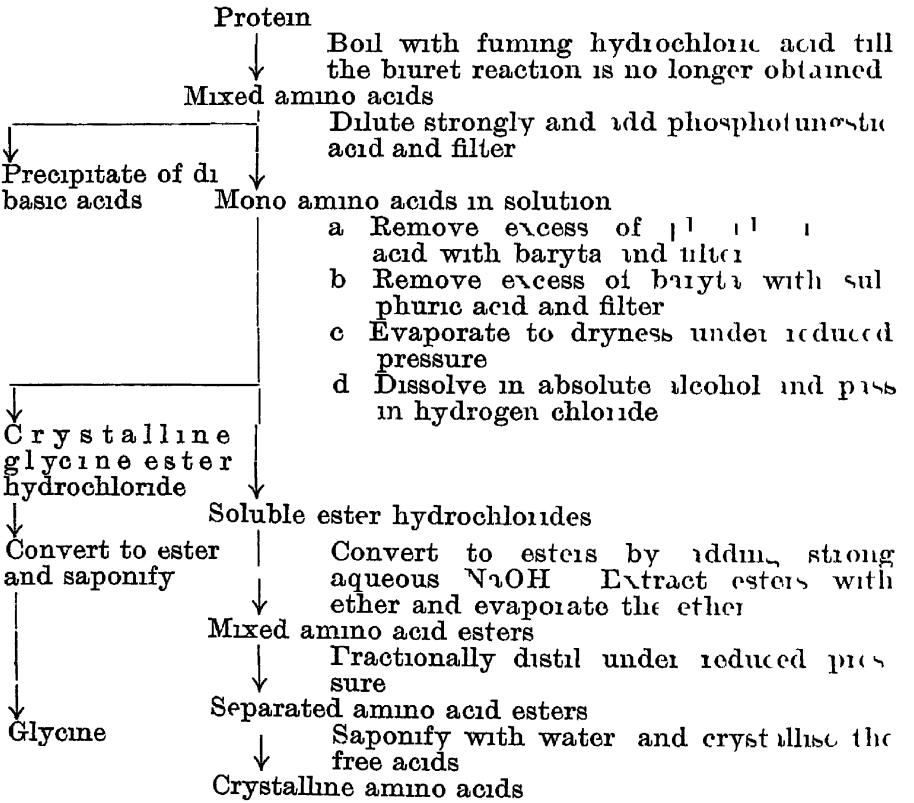
$$CH_2OH\ CHOH\ CHOH\ CHOH\ CHNH_2\ CHO$$

is obtained from many proteins particularly the mucins and mucoids It is not certain whether it is ever present as a unit in the molecule or whether it exists as an associated compound Galactosamine has also been isolated These amino sugars are responsible for the Molisch reaction

Fischer's Method of Protein Analysis

The final result of a complete protein hydrolysis is a mixture of substances all having very similar properties The difficulties of separating the individuals has already been mentioned The basic group (arginine lysine histidine and cystine) is readily separated in the form of an insoluble precipitate with phosphotungstic acid (Drechsel 1889 Kossel and Kutscher 1900) the acid group (aspartic glutaminic and hydroxyglutaminic acids) as the calcium salt (Foreman 1914) or the barium salt (Buxton and Schryver 1921) The mono amino mono carboxylic fraction is the most difficult to deal with and Fischer's use of the esters to separate the amino acids of the mono amino series was one

of those innovations of technique that marks a landmark in the history of science It was by the use of this method that reliable knowledge was obtained of the constitution of the protein molecule where previously only conjecture had been possible A summary of Fischer's analytical method is therefore given below



Although few new units were discovered by means of this technique Fischer Abderhalden Kossel Osborne and Levene and many other workers made by its use the first analyses of proteins that could be considered quantitative The knowledge of the constitution of the proteins was thus enormously increased and the evidence obtained supported the theory that amino acids are the only essential units of the protein molecule

Analysis revealed the units of the protein molecule bu gave no clue as to how they had originally been joined together *i e* it gave no evidence either as to the nature of the linkages or of the structural pattern of the molecule Following on the analyses of fully hydro lysed proteins many attempts were made to analyse par tially hydrolysed material in the hope of finding some one or several amino acids localised at some special stage of a fractional hydrolysis and so obtaining some light on the pattern The positive results of an enormous amount of work were however negligible free amino acids separated at every stage of hydrolysis and in no characteristic order Fischer therefore conceived the heroic plan of solving the problem of protein constitution by taking crystalline amino acids and of using them as starting points for the synthesis of more complex bodies some of which it was hoped might resemble the naturally occurring proteins

There are three types of linkage possible between amino acids (1) The direct linkage between two carbon atoms

$$\mathrm{R{-}C{=}C{-}R}$$

This can be rejected as a general protein link since trypsin which readily digests nearly all proteins does not attack it (2) the linl age between two carbon atoms by means of oxygen

$$\mathrm{R{-}C{=}O{-}C{=}R}$$

This is unlikely to be a general type since after accounting for the carboxylic groups proteins contain insufficient oxygen for the nur ber of linkages necess ary (3) fin ally the linkage of two carbon atoms by means of a nitrogen atom

$$\mathrm{R{-}C{=}N{-}C{=}R}$$

Thi linkage can exist in three forms as

(*a*) $\mathrm{—CH_2—NH—CH—}$

found in proline

(*b*) $-CH_2-NH-C(NH)-$

found in the guanidin nucleus of arginine

(*c*) $-CH_2-NH-CO-$

This type of linkage known as the peptide linkage would be formed by the condensation of an amino and a carboxylic group Fischer considered the possibility of this linkage occurring as the principal one in the protein molecule Its existence would account for two striking facts of protein chemistry firstly the very small amount of free amino nitrogen present in intact proteins and secondly its very large increase during hydrolysis Fischer therefore started his work on the condensation of the amino acids by coupling them by means of a peptide linkage

Synthesis of the Polypeptides

A polypeptide may be defined as a compound of known constitution formed by the condensation of two or more amino acids by means of a peptide linkage Compounds of this type *i e* di and tri peptides have also been found to occur naturally in tissues and have been isolated from the products of protein hydrolysis

Fischer carried out his work of condensing the amino acids by coupling two units by a carboxylic and an amino group *i e* by a peptide linkage He accomplished this synthesis by the use of acid chlorides under anhydrous conditions

(1) $CH_3\ COOH \longrightarrow CH_2Cl\ COOH \longrightarrow CH_2Cl\ COCl$
acetic acid monochloracetic acid chloracetyl chloride

(2) $CH_2Cl\ CO\ Cl + HNH\ CH_2\ COOH \longrightarrow$
chloracetyl chloride glycine

$CH\ Cl\ CONH\ CH\ COOH$
chloracetyl glycine

(3) $CH_2Cl\ CO\ NH\ CH_2\ COOH + 2NH_3 \longrightarrow$
chloracetyl glycine
$CH_2NH_2\ CONH\ CH_2\ COOH + NH_4Cl$
glycyl glycine

Glycyl glycine was the first dipeptide synthesised by Fischer but by using other acid chlorides other dipeptides could be made Fischer subsequently prepared the acid chlorides of the amino acids directly and from these synthesised the polypeptides The course of the reaction is summarised in the following equation

$CH\ NH_2\ CO\ Cl + H\ NH\ CH_2\ COOH \longrightarrow$
glycyl chloride glycine
$CH_2NH_2\ CONH\ CH\ COOH$
glycyl glycine

Fischer eventually succeeded in building up an octadeca peptide *i e* a chain containing eighteen amino acids and in 1916 Abderhalden and Fodor by making a polypeptide containing nineteen acid units created a record which few people will feel tempted to challenge

The synthetic polypeptides closely resemble in many of their properties the naturally occurring proteins They all give a biuret reaction The other colour tests are only given when a characteristic acid has been condensed into the peptide The higher polypeptides octa peptides and so on are precipitated by tannic acid and saturated ammonium sulphate and will not pass through a dialyser All the polypeptides are attacked by nitrous acid with the evolution of free nitrogen They are hydrolysed by acids and all alis yielding free amino acids most of them are hydrolysed by pancreatic trypsin none by pepsin They are all hydrolysed by the tryptases of the tissues and of micro organisms (For a further account of enzyme action on proteins see Chapter V)

The Nature of the Protein Molecule

The invariable presence of amino acids in the products of protein hydrolysis has been known for nearly fifty years Drechsel (1889 1891) realised the existence of ammonia and amino acids as units in the protein molecule Siegfried (1891) Hedin (1895 1896 1897) and Kossel (1896) showed that the same amino acids could be isolated from the products released by the acid or alkaline hydrolysis of different proteins Hopkins and Cole (1903) refer to the products of tryptic digestion of proteins as being characteristically of the nature of amido acids Although the possibility that the protein molecule is formed entirely by the condensation of amino acids is considered in these earlier papers the actual proof that these bodies are the only units essential to the structure is provided by Fischer s work on the synthesis of the polypeptides

The very close resemblance in properties between naturally occurring proteins and the higher synthetic polypeptides leaves no doubt that proteins are bodies formed by the condensation of amino acids largely by means of a peptide linkage The establishing of this generalisation is Fischer s great work His separation of amino acids by the esterification method nevertheless failed to account for more than 50 per cent of the original material and the possibility was not excluded that other types of unit might yet remain undiscovered Later workers have devised improved methods of analysis have obtained new amino acid units and improved yields of previously known units Their results however all go to confirm the theory that either amino acids or very closely allied substances are the only fundamental units in the protein molecule The later methods of protein analysis will be dealt with in the next chapter

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CHAPTER II

THE METHODS OF PROTEIN ANALYSIS

Methods of Hydrolysis—Classes of Amino Acids—Dakin's Butyl Alcohol Extraction Method—Foreman's Lead Salt Method—Kingston and Schryver's Carbamate Method—Amino Acid Derivatives—Colorimetric Methods of Estimation—Special Unit—Analysis by Nitrogen Distribution—the Method of Van Slyke—The Molecular Weights of Proteins—The Work of Troensegaard

Methods of Hydrolysis

It was shown in the previous chapter that a fuller knowledge of the constitution of the different proteins had to await the development of adequate methods of analysis. A number of new methods both for estimation and isolation of protein units have become available in recent years. In nearly every case the initial stage of the analysis is a hydrolysis of the protein which may be brought about by the action of an acid an alkali or a proteolytic enzyme. *Acid hydrolysis* opens up nearly all the linkages connecting the units in the protein molecule and reduces the protein almost completely to a mixture of free amino acids. It has the advantage of being a rapid process and the disadvantage that it leads to a destruction of all the tryptophane present together with much of the cystine. Both of these units as will be seen later are substances of immense biological importance. Under the action of the acid the tryptophane goes to form an insoluble black precipitate known as humin. The course of the reaction is obscure. According to Holm and Gortner (1920) it is largely influenced by the presence of aldehydes. In the absence of these the tryptophane does not contribute to the humin nitrogen and its nitrogen may

appear in the ammonia figure The cystine is largely trans formed into cysteine and partly destroy d by unknown reactions *Alkaline hydrolysis* leads to the formation of an insoluble residue of racemised protein with destruction of arginine and cystine and possibly some loss of histidine *Enzyme hydrolysis* is very slow *in vitro* and is never com plete Out of this choice of evils hydrolysis by acid is the method usually adopted for the general analysis of a protein

Classes of Amino Acids

The units obtained after hydrolysis may be classified as the basic or di amino fraction the di carboxylic fraction and the mono amino fraction including proline and hydroxy proline The di amino fraction is usually separated at some stage of the analysis as insoluble phosphotungstates The di carboxylic acids can be separated as their calcium salts by the addition of excess of lime and of alcohol to a con centration of 80 per cent This is the lime alcohol method of Foreman (1914) The mono amino fraction containing twelve or more possible units forms an analytical problem of much difficulty and it is in the separation of these that the greatest loss occurs Fischer s esterification method was devised for dealing with this group the most successful recent methods are due to the work of Dakin Foreman and Kingston and Schryver

Dakin's Butyl Alcohol Extraction Method

Dal in (1918) introduced the technique of separating the products of acid hydrolysis by continuous extraction with a solvent partially miscible with water Butyl alcohol is the most suitable solvent for this purpose The amino acids are not soluble in dry butyl alcohol but are readily soluble in moist butyl alcohol Only mono amino mono carboxylic acids proline and diketopiperazines are extracted by the

butyl alcohol The more highly ionised di basic and di carboxylic acids are left behind quantitatively in the aqueous solution

Dakin s method leads to the separation of the protein units into five groups These are —

(1) The mono amino acids extracted by butyl alcohol but insoluble in ethyl alcohol

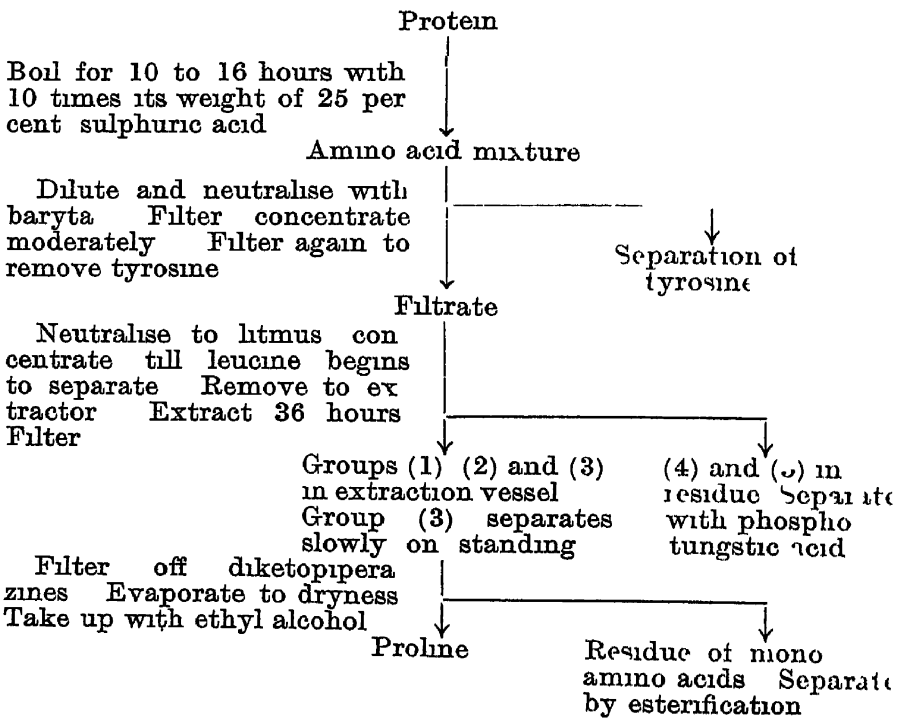
(2) Proline extracted by butyl alcohol and soluble in ethyl alcohol

(3) Diketopiperazines extracted by butyl alcohol but sparingly soluble in ethyl alcohol or in water

(4) Di carboxylic acids not extracted by butyl alcohol

(5) Di amino acids not extracted by butyl alcohol but separated from (4) by precipitation with phos photungstic acid

The method is summarised below —



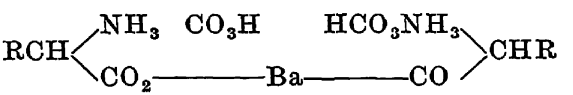
The great advantage of Dakin's method over Fischer's earlier one is that the mono amino acids are obtained dry and with practically no loss The yields obtained therefore correspond more nearly to the percentage derived from the original protein during the hydrolysis Dakin's figures for the constitution of gelatin are given in Table I column V (p 47) It can be seen that over 90 per cent of the protein has been recovered as free amino acids

Foreman's Lead Salt Method

Two other methods have been described by which the mono amino acids can be obtained in a satisfactorily dry condition previous to esterification These are the lead salt method of Foreman (1919) and the carbamate method of Kingston and Schryver (1924) In Foreman's method the di amino and di carboxylic acids are first removed from the mixed products of hydrolysis and the mono amino acids are converted into soluble lead salts by means of precipitated lead hydroxides and litharge Humin and unhydrolysed residues are filtered off and the solution of lead salts evaporated to dryness The lead salts can now be obtained in a thoroughly dry state and converted into their esters and the latter distilled without loss due to saponification This method gives high yields of the units Foreman's analysis of casein is given in Table I column IX

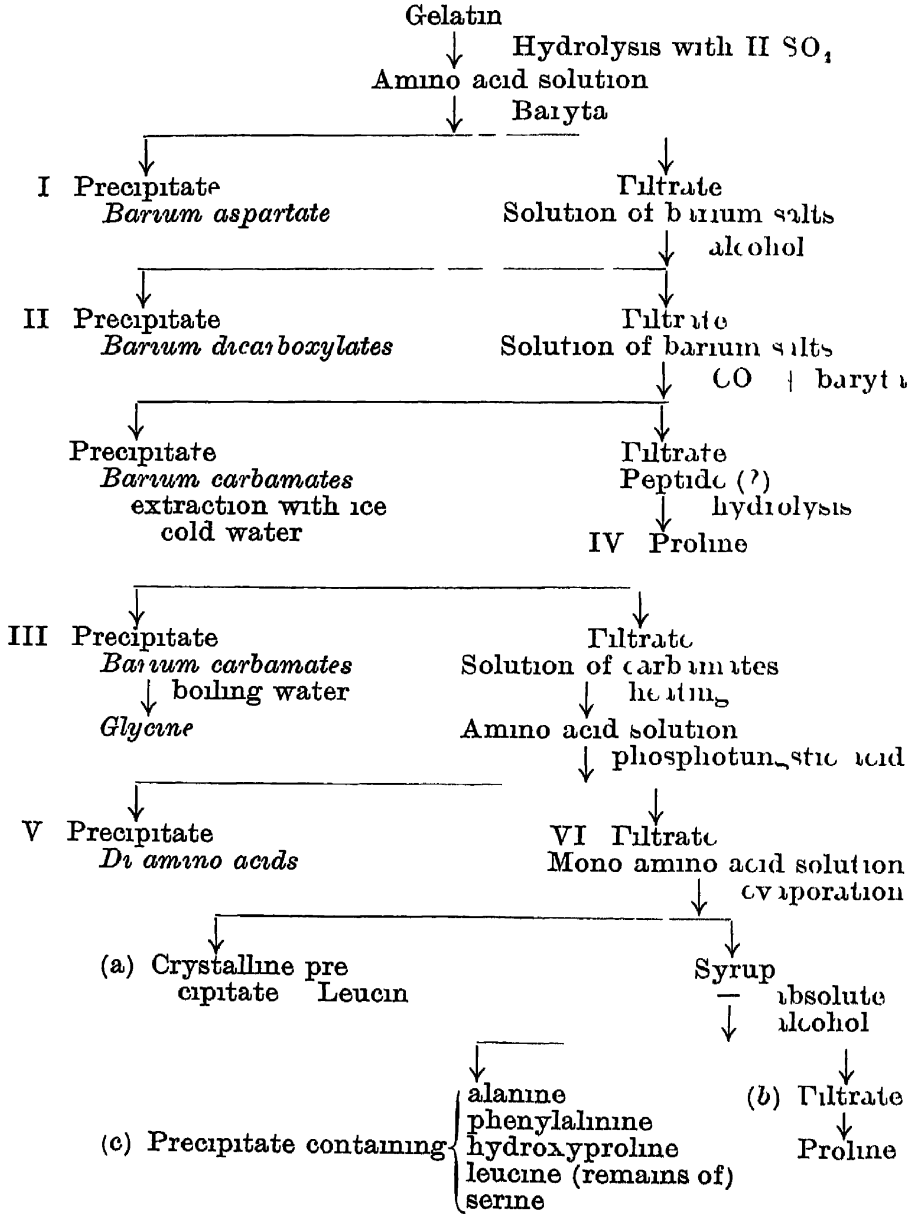
Kingston and Schryver's Carbamate Method

Kingston and Schryver make use of the property of amino acids when dissolved in aqueous alcohol of forming with barium hydroxide and carbon dioxide crystalline carbamates of the type



The method is said to be adaptable to small quantities of material and to give accurate estimations of glycine di

carboxylic and di amino acids proline and hydroxyproline A summary of the method is given below



Amino Acid Derivatives

Whilst the methods described above are available for a general analysis of a protein it sometimes happens that only the determination of some special unit is required In this case it is possible to make use of special methods adapted to the object in view The solubilities of the amino acids themselves do not for the most part differ very widely but the acids form a number of crystalline derivatives which in many cases have characteristic and low solubilities *The silver salts* were largely used for separation in the early days of protein chemistry Hedin (1895) separating arginine in this form The most important amino acid derivatives used recently for isolating and estimating particular units are given below

(1) *The Copper Salts* —Amino acids form crystalline copper salts some of which are only slightly soluble Harris (1923) uses this method to prepare cystine free from tyrosine

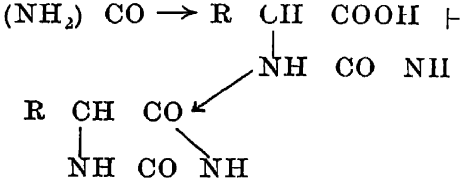
(2) *The Lead Salts* —In the distillation of the esters leucine and valine distil off together and the free acids cannot be separated by fractional crystallisation The lead salt of leucine and isoleucine is however much less soluble than the lead salt of valine Levene and Van Slyke (1909) separated the two acids by this means

(3) *H* —Both the amino acids themselves and the amino acid esters form hydrochlorides Glutaminic acid and histidine hydrochlorides have low solubilities and can be separated by fractional crystallisation (Frankel 1913) Glycine ester hydrochloride is insoluble in alcohol and is separated in this form in Fischer s method

(4) *Compounds with Mercury Salts* —Amino acids form crystalline compounds of varying composition with other mineral salts By means of a solution of mercuric sulphate in dilute sulphuric acid tryptophane cystine and tyrosine can be obtained as crystalline mercury derivatives of un known constitution Hopkins and Cole (1901) first used this

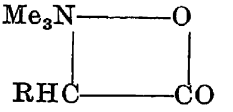
reagent for the isolation of tryptophane The mercury sulphate tryptophane derivative was decomposed with hydrogen sulphide and after removal of the sulphuric acid by baryta the tryptophane was recrystallised and determined by a direct weighing Onslow (1921 1921) determines tryptophane by estimating the nitrogen of the mercury sulphate precipitate

(5) *Urea Derivatives* —Amino acids combine with urea to form uramino acids which are readily converted into hydantoins on warming with dilute mineral acid



Dakin (1920) uses this method for determining hydroxy proline

(6) *The Betaines* —The amino acids can after methylation be converted into crystalline anhydrides the betaines



Engeland (1922) estimates proline by isolating it as the crystalline betaine of pyrolidin α carbonic acid and converting it into the aurichloride $C_7H_{14}NO_2$ $AuCl_4$ Accurate analyses can be made with less than a gram of material

Colorimetric Methods of Estimating Special Units

The separation of even a single unit from the products of hydrolysis of a protein is a tedious operation and frequently subject to considerable loss In recent years therefore methods have been sought to determine special units without isolation The chief of these methods are colorimetric Only certain units have been determined colorimetrically These are tryptophane cystine tyrosine and histidine Folin and

Denis (1912) describe two reagents for colorimetric work called by them the *phenol reagent* and the *uric acid reagent* respectively The phenol reagent is a mixture of sodium tungstate phosphomolybdic acid and phosphoric acid The uric acid reagent is a mixture of sodium tungstate and phosphoric acid

Tyrosine —The phenol reagent gives a blue colour with tyrosine Under certain rigorously defined conditions the depth of the colour is a measure of the concentration of the tyrosine These conditions are defined by Folin and Looney (1922) Tryptophane and phenolic bodies must be absent The method of determining tyrosine by the use of the phenol reagent has been adversely criticised by Furth and Fleisch mann (1922) who describe a method based on bromine absorption by the mono amino acid fraction

Cystine —Folin and Looney (1922) and Breeze Jones Gersdorff and Moeller (1924) use the uric acid reagent of Folin and Denis for estimating cystine Under carefully con trolled conditions the depth of the blue is a measure of cystine concentration Their determinations of cystine for a number of proteins are given in Tables I and II (pp 46–48) at the bottom of the columns Okuda (1924) has described a method of determining *cysteine* based on its power of absorbing bromine *Cystine* can also be determined after reduction with zinc dust and hydrochloric acid

Tryptophane —Furth and Lieben (1920) estimated trypto phane colorimetrically by a method founded on Voisenet s reaction which they showed to be specific for this unit The colour developed in the presence of formaldehyde sodium nitrite and concentrated hydrochloric acid was matched against a standard solution of gentian violet The propor tions of the reagents required to produce the maximum colour in any solution have to be found by trial and error

Folin and Looney (1922) estimated tryptophane (after the removal of the tyrosine) by the phenol reagent of Folin and Denis A more reliable method however seems to be the

method of May and Rose (1922) They find that by gently heating a protein in the presence of hydrochloric acid and *p* dimethylaminobenzaldehyde a blue colour develops the intensity of which is proportional to the tryptophane present In Tables I and II (pp 46–48) are given a number of tryptophane determinations made by Breeze Jones Gersdorff and Moeller (1924) using the method of May and Rose

Histidine —Koessler and Hanke (1919) describe a colorimetric method for the determination of histidine The method is based upon the interaction between the imidazole ring and *p* diazobenzene sulphonate The colour produced under very carefully defined conditions is matched against a colour standard prepared by mixing Congo red and methyl orange The method gives good results in the hands of its originators but has not yet been generally applied

Analysis by Nitrogen Distribution The Method of Van Slyke

A full hydrolysis of a protein and isolation of its units is a long tedious process and not very accurate In many cases too the elaborate detail is unnecessary Only a few of the amino acids can be determined colorimetrically Other methods have therefore been sought to determine the character of a protein without resort to a full amino acid separation The most successful of these first suggested by Hausmann (1899) and later brought to a high degree of technical efficiency by Van Slyke (1911) has been the method of determining the distribution of the nitrogen between the mono amino and the di amino groups It will be seen later that the food value of a protein can to a considerable extent be judged on the distribution of its nitrogen and the method therefore becomes of considerable economic importance

Van Slyke s method is based on the following interaction between free amino groups and nitrous acid

$$\begin{array}{l} R{-}CH\ NH_2 \\ \quad\ |\ \\ \quad COOH \end{array} + HNO \rightarrow \begin{array}{l} R\ CH\ OH \\ \quad\ |\ \\ \quad COOH \end{array} + H_2O + N_2$$

The amino groups are determined by the amount of nitrogen produced

The nitrogen in a protein can be regarded as distributed in the following manner —

Total N
- I Amide N
- II Di amino N + cystine N + Cystine N, Lysine N } Contain only amino N; Arginine Three quarters of N is non amino N; Histidine Two thirds of N is non amino N
- III Mono amino N + non amino N + A Mono amino N: glycine N etc, ½ tryptophane N, dicarboxylic acids N; B Non amino N: proline, hydroxyproline, ½ tryptophane N

In making an analysis by Van Slyke s method the protein is completely hydrolysed with strong hydrochloric acid and the nitrogen in the different groups is estimated by the following methods —

(1) The *total nitrogen* is determined on a portion of the hydrolysis solution by oxidising with concentrated sulphuric acid whereby the nitrogen is completely converted into ammonium sulphate Excess of caustic soda is added and the ammonia is distilled off into standard acid (Kjeldahl determination)

(2) *Fraction I and Humin Nitrogen* —Another portion of the hydrolysis solution is evaporated *in vacuo* to remove the excess of acid neutralised with magnesia and the *amide nitrogen* (I) is distilled off as ammonia

(3) A black residue remains in the hydrolysis flask This is filtered off and estimated for nitrogen by Kjeldahl s method This *humin nitrogen* is usually attributed to the tryptophane

(4) *Fraction II* —The filtrate from (3) is acidified and

phosphotungstic acid is added This separates groups II and III the former being in the precipitate the latter remaining in solution The precipitate is filtered off

(5) The precipitate is decomposed by barium chloride the barium phosphotungstate being filtered off

(6) *Fraction II —The total nitrogen of Fraction II* (a) is determined by a Kjeldahl estimation

(7) The *arginine N* is determined by boiling under a reflux condenser with strong sodium hydroxide Half the nitrogen of arginine is converted into ammonia and can be titrated Call the arginine nitrogen (b)

(8) *Cystine N* is calculated from an estimation of the sulphur as sulphate Call the nitrogen calculated for cystine (c)

(9) The *total amino nitrogen of Fraction II* is determined by the Van Slyke apparatus Call the value (d) (a) — (d) gives the non amino nitrogen of Fraction II This comes only from arginine and histidine From (a) — (d) subtract $\frac{2}{3}$ (b) The result is the non amino nitrogen of the histidine Multiplying this by two thirds gives *histidine N* Call this (f)

(10) *Lysine nitrogen* = (a) — (b) — (c) — (f)

(11) *Fraction III* —The total nitrogen of Fraction III is determined by Kjeldahl's method (g)

(12) The *amino nitrogen* of Fraction III is determined in the Van Slyke apparatus (h)

(13) Non amino nitrogen of Fraction III = (g) — (h)

The analyses of proteins made by Van Slyke's methods have given much valuable information on constitution especially for many proteins which are used as food materials either for man or his domestic animals The analysis though tedious is incomparably less arduous than a hydrolysis and separation of units The nitrogen determined in the fractions usually agrees to within 1 or 2 per cent of the total nitrogen of the intact protein The chief sources of error lie in the calculation of the cystine which it is now known is not the only sulphur containing group in the molecule and

the calculation of the lysine which carries the total error from three different experimental determinations Examples of analytical figures obtained for different proteins by Van Slyke s method are given in Chapter IV where the applica tion of the technique to the problem of protein identification is dealt with at length The constitutions of a number of typical proteins belonging to the different classes of both animal and vegetable proteins are given in Tables I and II pp 46–48 The figures have been compiled from results obtained by the use of the three classes of analytical methods described above namely by the isolation and direct deter mination of units by indirect colorimetric determinations of these and by determinations based on the distribution of the nitrogen The wide variety in the constitution of the proteins is well shown in the different columns of the tables

The Molecular Weights of Proteins

A full analysis of a protein and an estimation of its content of amino acids can be used to obtain an idea of the mole cular weight of the original protein Calculations of mole cular weights on amino acid content are based on three assumptions firstly that the amino acid used for the basis of the calculation was originally present in the protein and is not merely in the mass of hydrolysed material as the result of an admixture with an unrecognised impurity secondly that the determination of the amino acid is an accurate estimate of the original amount of the precursor present in the protein and finally that the result obtained is to be regarded as a minimum value only and subject to multiplica tion by a factor n where n may be any integer

In this way the writer (1920) calculated the minimum molecular weight of gelatine to be of the order of 10 000 Dakin s estimation of the histidine present was used as the foundation of the calculation Loeb (1922) by a similar calculation based on Dakin s figure for phenylalanine

obtained 11 800 A molecular weight obtained by a similar calculation is that of hæmoglobin calculated on the assumption that the molecule contains one atom of iron The value obtained is 16 000 Cohn Hendry and Prentiss (1925) have recently gathered together all the available evidence on the determination of special groups or atoms in certain proteins and have used these figures together with the combining weights for acids and alkalis obtained by titration and certain physico chemical measurements such as osmotic pressure determinations for estimating the molecular weights of proteins Their paper gives a full review of the literature They give a list of minimal molecular weights calculated to contain atomic or molecular equivalents of the following groups Iron (hæmoglobins) copper (hæmocyanins) sulphur sulphide sulphur phosphorus (casein) tryptophane tyrosine cystine histidine arginine and lysine

Protein	Minimal molecular weight	Protein	Minimal molecular weight
Gelatin	10 300	Egg albumin	33 800
Zein	19 400	Glutenin	36 300
Chadin	20 700	Fibrin	42 000
Hæmocyanin (limulus)	22 700	Serum albumin	44 000
Bence Jones protein	24 500	Hæmoglobin (dog)	60 000
Edestin	29 000	Serum globulin	81 000
Hæmocyanin (octopus)	33 500	Casein	192 000

It should perhaps be mentioned here that some workers do not regard the proteins as chemical individuals but rather as constant composition mixtures They consider that the groupings in the protein are not held in association by the ordinary valency bonds of chemical combination *i e* by electronic interchange between two atoms but by residual electric fields On this theory of protein constitution the disintegration of a protein into its units is not a chemical degradation but a physical separation and the calculation

of molecular weights for proteins is a proceeding of some what dubious value

The Work of Troensegaard

A worker with novel and interesting views on the constitution of the protein molecule is Troensegaard (1920 1923) He considers that the analysis of a protein by means of hydrolysis gives misleading results His method is to acetylate the protein by means of acetyl chloride in acetic acid purify the acetylated product by extraction with organic solvents and then hydrogenate by metallic sodium in amyl alcohol Water is rigorously excluded during hydrogenation The reduced protein is fractionated by a series of organic solvents He obtained a number of substances containing pyrrolin and pyrrolidin rings which he regards as fundamental units in the molecule Troensegaard s work is suggestive and stimulating but his view that the amino acids are artefacts rather than natural units does not harmonise with two important biological facts—first that the alimentary enzymes decompose proteins with the production of amino acids and secondly that amino acid mixtures and these only can be substituted for proteins in the diet of mammals

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CHAPTER III

THE DIFFERENT CLASSES OF PROTEINS

Plant and Animal Proteins—Biological Classification of the Proteins—Chemical Classification of the Proteins Group I — The Simple Proteins the Protamines the Histones the Albumins and the Globulins the Scleroproteins the Keratins the Gliadins the Glutelins Group II —The Conjugated Proteins the Phosphoproteins the Hæmoproteins the Muco proteins the Nucleoproteins Group III The Derived Proteins

Plant and Animal Proteins

THE chemical composition of all proteins is essentially similar whatever their origin may have been In their pro perties however they differ considerably and these differences are broadly associated with biological differences in origin and function Proteins in association with water constitute the protoplasm of all living cells both animal and vegetable The bacteria structurally among the simplest organisms known consist of little protoplasmic masses without a well defined nucleus and with no structurally distinct cell wall The bacteria however are a very special type of living organism All other unicellular organisms whether plant or animal are differentiated into the cytoplasm and a definite cell structure the nucleus which has been proved to control the living activities of the cell The proteins of the cytoplasm belong always to the classes known as albumins and globu lins They are very sensitive to changes in their physical and chemical environment readily soluble under many conditions and not easy to isolate in an unchanged condition The nucleus is composed largely of proteins in association with nucleic acid probably as salts Nucleic acid itself is a very complicated condensation product of purine and

pyrimidine bases phosphoric acid and a sugar group It can be obtained from both plant and animal tissues Cell nucleus and cell cytoplasm are very similar systems in both animal and vegetable kingdoms All living organisms therefore start with a common chemical basis but with the evolution of definite plant and animal characteristics two widely divergent lines of chemical evolution appear The evolution of the multicellular organism makes possible both increased size of body and the differentiation of specialised tissues This increase in complexity necessitates the development of special accessory organs for support and for other purposes The plant uses carbohydrates as the material out of which to manufacture special substances for special needs—cellulose lignin mucilage etc The animal on the other hand uses protein It is outside the scope of this book to consider whether these two lines of chemical evolution are bound up with the two methods by which plant and animal obtain their energy and the form in which they take their food The green plant uses as food the simplest chemical substances—water carbon dioxide ammonia etc —and obtains its energy from the sunlight The animal uses as food material in a higher state of chemical complexity—sugars amino acids etc —and obtains its energy by the oxidation of these The green plant uses energy in the synthesis both of protein and of carbohydrate but owing to the higher proportion of oxygen in the carbohydrate molecule the amount of energy thus made latent is less in a skeleton made of cellulose than in one made of collagen For the animal feeding on plant or other animal tissues a store of chemical energy is available in its food and this may possibly account for the apparent extravagance with which protein material is transformed to serve all kinds of mechanical purposes and is thus lost from the main cycle of metabolism

It follows from these two lines of development that plant proteins are comparatively similar and belong only to a few classes all of which are found as the contents of cells They

form cytoplasm and reserve food (aleurone grains) in the cells of the adult plant and in seeds There are only five classes of plant proteins—albumins globulins nucleoproteins gliadins and glutelins the two latter being confined to the seeds of cereals An account of the vegetable proteins is given in Osborne s The Vegetable Proteins

Animal proteins on the other hand differ widely in their properties and form a large number of groups They may like plant proteins be intra cellular when they are part of the living cell or they may be extra cellular when they have been transformed to serve some special function This may be as a system of supporting tissues as in the connective tissue proteins (the *sclero proteins*) or as a protective covering for the skin (the *keratins*) or as a lubricating material to assist the passage of food in the alimentary canal (*muco proteins mucins*) or as a food material for the young (*phos phoproteins*) or as carriers of the respiratory pigment (*hæmoproteins hæmoglobin*) Besides the albumins and globulins of the cytoplasm two other classes of intra cellular proteins associated specially with the generative cells have been found in the animal kingdom—the *histones* found in gland cells and the immature generative glands and the *protamines* found only in the ripe sex cells The intra cellular proteins probably take an active part in the cycle of metabolism the extra cellular proteins are in relation to the animal which produced them products without any further function in the main metabolic cycle In the former group are the protamines histones albumins and globulins and it is an interesting relationship that the more closely associated a protein is with cell proliferation the greater its content of di amino acids especially arginine This is especially the case with the protamines of the ripe sperm head a cell which has no other function beyond inducing cell division and regulating the distribution of material in the newly formed cells of the early embryo The keratins and collagens have no metabolic activity

and it seems more than a coincidence that these inert proteins have out of all classes of proteins the lowest content of di amino acids The great variety of characteristics found among animal proteins has lead to many of them being adapted for industrial purposes—silk wool hides etc are all of great economic importance and most proteins of technical industrial importance are of animal origin

A classification of the proteins based on purely biological principles is given below

Biological Classification of the Proteins

(A) **Plant Proteins**

I *Intra cellular Proteins*

(1) Albumins, (2) Globulins } Cytoplasm proteins
(3) Gliadins
(4) Glutelins
(5) Nucleoproteins

} Food reserve in seeds

(B) **Animal Proteins**

I *Intra cellular Proteins*

(1) Albumins, (2) Globulins } Cytoplasm proteins and as food store for young
(3) Histones—present in gland cells and in immature generative cells
(4) Protamines—present in ripe generative cells
(5) Nucleoproteins

II *Extra cellular Proteins*

α The Connective Tissue Proteins or Scleroproteins

(1) The Collagens and Gelatins
(2) Reticulin
(3) Elastin

} Supporting or skeletal material

β The Epidermal Proteins

(1) The Keratins—hair wool etc
(2) Silk

} Protective material

γ The Mucoproteins (Glucoproteins)

(1) The Mucins
(2) The Mucoids
} Slimy material with a lubricating and possibly sometimes a supporting function

δ Pigment carrying Proteins or Hæmoproteins

(1) The Hæmoglobins
(2) The Hæmocyanins
} Materials with a respiratory function

ε Food Proteins for the young animal

(1) Phosphoproteins—present in milk and egg yolk

Chemical Classification of the Proteins

A chemical classification of the proteins was drawn up by a Committee of the Physiological Society in 1907 (1) and by the Committee of the American Physiological Society in 1908 (2) The schemes adopted by the two Committees were very similar and are in use at the present day It is acknowledged however that though they may have been the best classification possible at the time they are not really adequate and need revision They will doubtless ultimately be superseded but meanwhile the different groups to which their use leads have a certain individuality both in chemical character and biological origin The schemes provided for the division into three main groups (I) Simple proteins formed from amino acids only (II) Conjugated proteins formed by the association of a protein with some other group (III) Derived proteins regarded as successive steps in the hydrolytic breakdown of proteins The first group is subdivided according to the solubilities of its members in various reagents the second according to the nature of the associated non protein group (prosthetic group) and the third into somewhat more or less ill defined groups that correspond approximately to the degree of degradation that may be supposed to have occurred The recent work of Wasteneys and Borsook (1924) seems to have brought some much needed precision into this last group

GROUP I —SIMPLE PROTEINS

	O	S l b lty	Ch t t P p ty
1 *The pı otamınes*	Anımal cells only	Soluble ın dılute acıds precıpıtated by am monıa	Stıongly basıc
2 *The hıstones*			Moderately basıc
3 *The albumıns*	Anımal and vege table cells	Soluble ın wateı and salt solutıons acıds and alkalıs	Coagulated by heat Soluble ın wateı Precıpıtated by saturated ammonıum sul phate
4 *The globulıns*		Soluble ın salt solutıons acıds and alkalıs A few soluble ın wa'eı	Mostly coagulated by heat Precıpıtated by half satu ıated ammonıum sulphate
ɔ *The connective tıssue pıo teıns (sclei opı oteıns)*			
(a_1) *The collagens*	Secıeted by anımal cells	Insoluble ın wateı acıds alkalıs and salt solu tıons	Swell ın dılute acıds and alkalıs yıeld gelatın on boılıng
(a_2) *The gelatıns* (de rıved protem)	Derıved fıom the collagens	Insoluble ın cold wateı soluble ın hot wateı Fauly soluble ın acıd and alkalı	Swell ın dılute acıds and alkalıs Solutıons ın hot wate set to a jelly on coolıng
(*b*) *Elastın*	Secreted by anımal cells	Insoluble	Does not swell Dıgested by trypsın
(*c*) *Retıculın*			Does not swell
6 *The keı atıns*			Veıy re ı tant to enzyme actıon
7 *The gliadıns*	Ceıeal eeds only	Soluble ın dılute acıd oı alkalı	Soluble ın 75 pei cent alcohol
8 *The glutelıns*		Insoluble ın wateı Sol uble ın dılute alkalı	Swell ın dılute acıds and al l alıs Form coherent dough wıth water

GROUP II —THE CONJUGATED PROTEINS

9 *The phosphoproteins*	Secreted by animal cells	Soluble in dilute alkalis	Contain phosphoric acid as part of the protein molecule It can be split off by the action of an enzyme phosphoric acid esterase
10 *The hæmoproteins*	Animal origin	—	Associated with respiratory pigment
(*a*) *The hæmoglobins*	Vertebrate blood		hæmatin containing iron in Vertebrates and a blue pigment containing copper in Invertebrates
(*b*) *The hæmocyanins*	Invertebrate blood		
11 *The mucoproteins*			
(*a*) *The mucins*	Secreted by cells of the animal intestine	Soluble in water and dilute alkalis	Slimy water holding bodies in which the protein is associated with a carbohydrate glucosamine
(*b*) *The mucoids*	Present in cartilage tendons etc	—	Viscous bodies in which the protein is associated with galactosamine
12 *The nucleoproteins*	Animal and vegetable cells	—	The protein is associated with nucleic acid

GROUP III —DERIVED PROTEINS

1 *Metaproteins*	Precipitated at *p*H 6 0
2 *Proteoses*	Not precipitated at *p*H 6 0 but completely precipitated by saturation with Na_2SO_4 at 33
3 *Peptones*	Not precipitated by above conditions but precipitated by tannic acid
4 *Peptides*	Not precipitated by above conditions but precipitated by alcohol
5 *Amino acids*	

Group I —The Simple Proteins

The subdivision of this group has been founded on varying solubilities Proteins have both acidic and basic groups and according to which of these two preponderates so will the solubility vary the more basic proteins dissolving more readily in acids than in bases and *vice versa* In solutions the hydrogen ion concentration of which has been so adjusted that basic and acidic groups are at the same ionisation potential there is found a region of minimum solubility The hydrogen ion concentration at this point is called the iso electric point of the given protein The iso electric point of the basic pro teins is in weakly alkaline solutions of the acidic proteins in weakly acid solutions Classification by solubilities then is in the first place a classification based on the value of the iso electric point which depends in its turn on the constitution of the molecule The basic proteins are the protamines and the histones These groups especially the former are chemi cally notable for their high content of di amino acids which may form from 20 to 90 per cent of the whole molecule The groups that may be considered approximately neutral are the albumins and globulins and gelatin The two former have a fairly high content of di amino acid precursors but these are balanced in the molecule by an equally high content of dicarboxylic groupings In gelatin and the other sclero proteins this balance is maintained but the greater part of the molecule consists of the simpler mono amino groupings glycine and leucine Proteins of an acidic character are the phosphoproteins which have a high content of di carboxylic groupings On the system of classification at present in use the phosphoproteins are placed in the next group (II The Conjugated Proteins) A new classification of the proteins based on the value of the iso electric point would have many advantages Cohn (1922) has shown that this physical constant can be used for identification The albumins and globulins differ by the relative ease with which

the latter can be salted out of solution The sclero proteins and the keratins were formerly classed together on account of their insoluble character but they differ very markedly in chemical composition in their behaviour towards acids and bases and in their reactions towards enzymes These facts suggest that the insolubility required by their static function in the organism has been achieved by different means in the two different groups

(1) *The Protamines* The protamines are animal proteins They have a comparatively simple composition and are formed very largely from the basic amino acids particularly arginine The constitution of a typical protamine is given in Table I Column I (p 46)

The preponderance of basic groups which the protamines contain gives them as might be anticipated a strongly basic character They are readily soluble in dilute acids and are precipitated from solution by ammonia Like all proteins they are soluble in strong alkalis *e g* sodium hydroxide solutions

The protamines form the bulk of the proteins in ripe sperm cells and ova It will be seen later (see Chapter VI) that the basic amino acids are of very great importance in the growth and development of the young animal The fact that the protoplasm of the germ cells consists mainly of protamines is therefore not without significance

Characteristic protamines are salmin prepared from the ripe roe of the salmon clupein from the herring etc The protamines are valuable foods but their economic importance is not great since the supply is limited

(2) *The Histones* —These are also of animal origin They are basic in character though not so strongly basic as the protamines They are readily soluble in dilute acids precipitated by ammonia but again soluble in sodium hydroxide They contain a high proportion of basic di amino acids but also a considerable amount of mono amino groupings They occur in unripe germ cells in the red blood corpuscles and

in the lymphoid tissue notably the thymus They may therefore be regarded as connected with metabolic activity though not with that intense activity peculiar to the developing embryo

The constitutions of the thymus histone and of globin the histone from hæmoglobin are given in Table I Columns II (*a*) and (*b*) The histones though valuable food materials are not sufficiently available to have any special economic importance

(3) and (4) *The Albumins and the Globulins* —These two groups often called the native proteins form the bulk of the protein content of the protoplasm of all adult cells whether animal or vegetable They are also found as a food material in milk eggs and seeds In vertebrate animals they are present in the blood The albumins are soluble in acids alkalis distilled water and salt solutions They seem to have for proteins comparatively small molecules for their solutions require saturation with ammonium sulphate before they can be salted out and they remain soluble in saturated magnesium sulphate solutions Egg albumin and serum albumin have both been obtained crystalline The globulins differ from the albumins by being salted out of solution by half saturation with ammonium sulphate or saturation with magnesium sulphate The animal globulins exist in two forms euglobulin insoluble in water and pseudoglobulin soluble in water A number of the vegetable globulins *e g* edestin have been obtained crystalline The iso electric points of albumins and globulins lie over a range of from *p*H 5 5 to 4 7

The chief chemical characteristic of these two groups is that the molecule consists of a small quantity each of a large number of amino acids *i e* there is no preponderating amount of any one or two constituents Basic amino acids are present but are balanced by the content of di carboxylic groupings

The biological importance of albumins and globulins in

cells appears to lie in their extreme sensitiveness to physical conditions The degree of dispersion in solution of serum globulin for instance is very greatly affected by hydrogen ion concentration and by salt content The movement of water into and out of cells is probably regulated largely by the response of the cell proteins to changes in reaction and salt content But the outstanding character of albumins and globulins is that they can be *coagulated by heat* This change is irreversible A similar change is brought about by strong alcohol The death of cells also is accompanied by an irreversible coagulation of the proteins and it may safely be assumed that life in the cell is associated with the maintenance of the uncoagulated condition of the proteins

The vegetable albumins differ from the animal albumins by being more readily salted out of their solutions a few of the former being precipitated by saturation with magnesium sulphate Vegetable albumins occur in small quantities in seeds

Vegetable globulins differ from animal globulins by being less readily salted out of their solutions a few remaining in solution with full saturation with magnesium sulphate They are also much less readily coagulated by heat a few showing no apparent change after boiling The bulk of the proteins of seeds are globulins

The proteins of the cytoplasm of the adult green plant have been very little studied Proteins from the leaves of the alfa alfa plant from spinach and from *Zea mays* have recently been isolated by Chibnall (1924) and Chibnall and Nolan (1924) These proteins which resemble each other closely are coagulated irreversibly by heat and strong alcohol They are insoluble in water (minimum solubility between *p*H 4 0 and 4 6) soluble in dilute acid and alkali but differ from all known proteins by being precipitated from their solutions by traces of salts They are probably closely allied to the globulins

Animal albumins form about a third of the protein of

TABLE

The Animal

Column	I	II		III		IV	
Class	Protamin	Histon		Albumin		Globulin	
		(a)	(b)	(a)	(b)	(a)	(b)
Protein	Salmin	Thymus Histon	Globin from Hæmoglobin	Lactalbumin (whole)	Serum albumin (horse)	Blood Fibrin	Serum globulin (horse)
Reference	Kossel & Dakin (1904)	Abderhalden & Rona (1904)	Abderhalden (1903)	Jones (1924)	Abderhalden (1903)	Hewlett & Sharpe (1911)	Alderhalden (1905)
Glycine	—	0·5	—	0·37	0	+	3·25
Alanine	+	3·5	4·2	2·41	2·7	+	2·22
Valine	4·3	—	—	3·30	—	5·6	—
Leucine	+	11·8	29·0	14·03	20·48	9·5	18·70
Isoleucine	—	—	—	—	—	1·0	—
Phenylalanine	—	2·2	4·2	1·25	3·1	4·9	3·84
Tyrosine	—	5·2	1·3	1·95	2·1	4·2	2·9
Serine	7·8	—	0·6	1·76	0·6	—	—
Cystine	—	—	0·3	1·73	4·23	0·6	0·7 (1·5)
Proline	11·0	1·5	2·3	3·76	1·04	2·7	—
Hydroxyproline	—	—	1·0	—	—	—	—
Aspartic acid	—	—	4·4	9·30	3·12	2·2	2·4
Glutamine acid	—	0·5	1·7	12·89	7·7	8·0	8·9
Hydroxyglutamic acid	—	—	—	10·00	—	—	—
Tryptophane	—	—	+	+	+	0·8	+
Arginine	87·4	15·5	5·4	3·47	4·9	6·1	3·9
Lysine	0	6·9	4·3	9·87	13·2	3·7	2·2 (8·9)
Histidine	0	1·5	11·0	2·61	3·4	0·8	2·8
Ammonia	—	—	—	1·31	1·2	—	1·75
	110·5	49·1	69·7	79·93	67·8	46·1	—

Colorimetric determinations Jones Gersdorff and Mueller (1924) and Furth and

Tryptophane	—	1·1	0·0	2·69	1·3	—	4·4*
Cystine	—	—	—	3·91–4·25	—	—	—
Total Sulphur	—	—	0·420	—	1·930	—	1·110
Cystine Sulphur	—	—	0·200	—	1·280	—	0·630

I

Proteins

V C t Pr t ( ) G l t	V (*b*) Elast	VI K t ( ) S lk Γ b	VI (*b*) H rs h	VII Ph ph p t C g	VIII T Pr t ( ) Ch k M l	VIII (*b*) H l b t	VIII ( ) O ʂ l
D k (19 0)	Abd h ld & S h tt l lm (1904) Sw t (1894) I l & I t h (1901)	Abd h ld (19)	Abd h ld & W ll (1905) A g (1907) B ht l (1907)	F m (1919)	O b & H yl (1908)	O b & H l (1908)	O b rn & J (1909)
25 5	25 8	40 5	4 7	0 45	0 68	0 0	2 1
8 7	6 6	25 0	1 5	1 85	2 28	?	3 7
0 0	1 0	—	0 9	7 93		0 79	0 8
7 1	21 4	2 5	7 1	9 7	11 19	10 33	11 7
0 0	—	—	—	—	—	—	—
1 4	3 9	1 5	+	3 88	3 53	3 04	3 2
0 0	0 4	11 0	3 2	4 5	2 16	2 39	2 2
0 4	—	1 8	0 6	0 5	?	?	?
0 0	—	—	8 0	—	—	—	—
9 5	1 7	1 0	3 4	7 63	4 74	3 17	5 8
14 1	—	—	—	+	—	—	—
3 4	+	—	0 3	1 77	3 1	2 73	4 5
5 8	0 8	0 0	3 7	21 77	16 48	10 13	15 5
0 0	—	—	—	—	—	—	—
0 0	—	—	—	1 5	—	—	+
8 2	0 3	1 5	4 5	3 81	6 50	6 34	7 5
5 9	+	0 85	1 1	7 62	7 94	7 45	7 6
0 9	—	0 75	0 6	2 5	2 47	2 55	1 8
0 4	—	—	—	1 6	1 67	1 33	1 1
91 3	61 9	86 4	39 0	(Phosphorus = 0 85) 75 0	62 5	50 7	67 5

Lieben (1920)		*Sulphur and Cyst ne Si lphur* (Osborne 1902)				(F sh)	
0 0	0 0	0·0*	—	2 20	—	1 °5	—
0 15–0 31	—	—	—	0 26	—	1 32	—
—	—	—	4 5	0 800	—	—	—
—	—	—	—	0 101	—	—	—

TABLE II

The Vegetable Proteins

C l m Cla I t R f	I Alb m ns L (Wh t) O b & Cl pp (1906)	II Gl b l ( ) Ed t (H p) Abd h ld (190 3)	(*b*) Sq h S d O b & Cl pp (1907)	( ) Gly (S y b) O b & Cl pp (1907)	III Gl d ( ) Wh t gl d O b & Cl pp (1906) O l & (t (1911)	(*b*) / (m) O b & Cl pp (1907 8) O l & I III (1910)	IV Gl t l Gl t l (wl t) O b & Cl III (1906)
Glycine	0 9	3 8	0 6	1 0	0 0	0 0	0 9
Alanine	4 5	3 6	1 7	—	2 0	9 8	4 7
Valine	0 2	+	0 3	0 7	3 4	1 9	0 2
Leucine	11 3	20 9	7 3	8 ∪	6 6	19 6	6 0
Isoleucine	—	—	—	—	—	—	—
Phenylalanine	3 8	2 4	3 3	3 9	? 4	6 6	2 0
Tyrosine	3 3	2 1	3 1	1 9	1 2	3 6	4 3
Serine	?	0 3	—	—	0 2	1 0	0 7
Cyst ne	—	0 3	0 2	—	0 5	—	0 02
Proline	3 2	1 7	2 9	3 8	13 2	9 0	4 2
Oxyproline	—	2 0	—	—	—	—	—
Aspartic Acid	3 4	4 5	3 3	3 9	0 6	1 7	0 9
Glutamimc Acid	6 7	6 3	12 4	19	43 7	26 2	23 4
Tryptophane	+	+	+	—	1 0	0 0	+
Arginine	5 9	11 /	14 4	1	3 ?	1 6	4 7
Lysine	2 8	1 0	2 0	2 7	0 ?	0 0	1 9
Histidine	2 8	1 1	2 6	1 4	0 6	0 8	1 8
Ammonia	1 4	—	1 6	2 6	5 ?	3 6	4 0
	50 2	61 7	55 9	55 0	84 0	85 4	9 72
Colorimetric determinations (Jones Gersdorff and Mu eller 1924)					*Sulphur* (Osbor ne 1902)		
Tryptophane	4 76	2 48	3 01	1 66	0 70–1 09	0 0	1 72
Cystine	3 29	0 97	1 38	1 12	1 42–1 76	0 8∪	1 46
Total Sulph	—	0 880	—	0 710	1 0?7	0 600	—
Cystine Sulphur	—	0 347	—	0 320	0 619	0 212	—

many cells and body fluids for example muscle fibres serum white of egg etc the remaining two thirds being globulins Animal globulins are very sensitive to physical conditions and can only be isolated with the greatest difficulty in an unaltered condition

The economic importance of the albumins and globulins lies in their high food value

Characteristic animal albumins and globulins are those from serum lactalbumin and lactoglobulin from milk ovalbumin and ovoglobulin from egg white myosin and paramyosinogen from muscle fibrinogen (globulin) from blood or plasma The constitutions of two animal albumins are given in Table I column III of two globulins in column IV and of three mixed tissue proteins in column VIII

Among vegetable albumins are legumelin from peas leucosin from wheat Among the vegetable globulins are edestin from hemp seed the globulin of pumpkin seed (both of which have been obtained crystalline) legumin from peas glycinin from the soya bean etc The constitution of a vegetable albumin is given in Table II column I and of three vegetable globulins in Table II column II

(5) *The Connective Tissue Proteins of Animals* (*Scleroproteins* (*a*) *The Collagens and the Gelatins*—The collagens differ from the four classes previously considered since they are never found inside cells but are laid down externally in the form of long fibres Collagens are peculiar to the animal kingdom They differ from the proteins of the cytoplasm by the loss of their solubility and most of the other characteristic physical properties of the proteins of the active cells This insolubility and increased stability are required by their static function in the organism They differ considerably in chemical composition from the albumins and globulins a fact which suggests that they are the products of cell secretion *i e* that they are synthesised by cells and are not merely cell substances transformed by additional internal linkages

In the vertebrates collagen fibres form a large part of the connective tissue of the body They are known histologically as white fibres They form a closely woven tissue of fine fibres and fibre bundles which in the form of thin sheets wrap round and penetrate the various organs of

the body and hold not only individual organs but also the whole body together In places *i e* at the end of the muscles the fibres run together to form the tendons The collagen fibres in their function of holding together form a wrapping tissue over the whole body *i e* they form the skin Cartilage also is composed largely of a collagen chondro collagen The function of the collagen is mainly that of supporting and padding tissue The supporting system may be reinforced locally by the deposition of calcium salts forming the bones The part played by connective tissue in maintaining the form of organs may be very important The outstanding feature of the collagens is their insolubility in water and salt solutions either hot or cold They swell in dilute acids and alkalis but do not appear to dissolve except in strong solutions (see Porter 1922 Merrill 1924)

In concentrated boiling acids or alkalis the collagens are hydrolysed giving a mixture of amino acids They are also hydrolysed by pepsin but not by trypsin or only very slowly After peptic action and after boiling the collagen residues are readily digested by trypsin

If hydrolysed gently by means of high pressure steam or boiling dilute acid collagen is converted into gelatin a protein never present in the living animal body and therefore strictly to be classified as a derived protein The chief characteristic of gelatin is its power of making heat reversible gels Gelatin has been obtained crystalline by Bradford (1923) Pure gelatin differs in many of its properties from crude gelatin It is only very slightly soluble in even boiling water Its iso electric point is at *p*H 4 6 and its solubility and degree of dispersion in solution increase rapidly on either side and in dilute salt solutions In fact in its physico chemical properties it closely resembles the globulins (see Jordan Lloyd 1921) In view however of the striking difference in constitution between gelatins and globulins and the fact that the former are not coagulated either by heat or by alcohol there seems insufficient justification for grouping

them together as suggested by Kingston and Schryver (1924) The chemical constitution of gelatin is shown in Table I column V The gelatins and also the collagens from which they are derived are conspicuous for their high content of glycine the simplest of all the amino acids and one which is very easily synthesised in the animal body They have a low content of the basic amino acids and correspondingly low content of the dicarboxylic acids whilst tryptophane cystine lysine and tyrosine four amino acids of great individual importance in animal metabolism are missing from their constitution For this reason the collagens and the gelatins have a very low food value Their economic importance lies in the two great industries of leather making and glue and gelatin manufacture Ossein the protein of bones is very closely allied to the collagen of the skin and is also used in the manufacture of gelatin and glue

(*b*) *Elastin* —Connective tissue consists not only of white fibres but also of so called yellow fibres These are formed of a protein known as elastin Elastin like collagen is to be regarded as an end product of cellular activity It is insoluble in hot or cold water cold dilute acids and alkalis and salt solution It does not swell in acid or alkaline solutions It is rapidly digested by trypsin and is hydrolysed by hot acids and alkalis Constitutionally elastin is somewhat similar to gelatin It yields large quantities of monoamino acids chiefly glycine and leucine and only traces of the more basic units The hydrolysis of elastin is shown in Table I column V (*b*)

(*c*) *Reticulin* —A remarkable tissue has been described by Mall (1896) and called the reticulum or reticular tissue This forms an exceedingly fine filamentous network penetrating the cellular organs connective tissue and skin and apparently forming a framework for a great deal of the cellular tissue in the animal body Siegfried (1902) has isolated this tissue from the liver of the dog and Miss Madge Kaye (unpublished work) a similar tissue from skin The isolated

products are proteins which have been called for convenience reticulin Reticulin from skin gives the biuret reaction and is rapidly attacked by pepsin and more slowly by bacterial tryptases It is not attacked in its fresh condition by pancreatic trypsin Its most remarkable characteristics are its resistance to the action of chemical reagents It is at least structurally unaffected by boiling water boiling dilute acids and alkalis and can withstand the action of cold concentrated acids and alkalis for many hours The molecular linkages in reticulin are at present only a subject for speculation Reticulin is an example of a protein transformed into a highly resistant chemically inactive material Photographs of skin sections showing unswollen reticular bands among collagen fibres swollen by acid are given in a paper by Kaye and Jordan Lloyd (1921)

Elastin and reticulin only exist as exceedingly filamentous tissues permeating the animal body Chemically they are of considerable interest and biologically their importance must be very great but their industrial importance is difficult to assess

(6) *The Keratins* — This group of animal proteins is the product of epidermal activity and includes the outer surface of the skin and such epidermal organs as scales feathers hair wool horns hoofs and nails and epidermal products such as silk The keratins are remarkable for their power of resisting many chemical reagents They are insoluble in water and dilute acids and alkalis They are slowly hydrolysed by concentrated boiling acids they are fairly readily hydrolysed by strong alkalis in the cold The keratins are remarkable for the rapidity with which they are attacked by the sulphides of the alkalis and the alkaline earths The reaction which takes place is not fully understood but Marriott (1925) has shown that some interaction occurs in the case of hair between the sulphide present in the hair and the divalent sulphide ions in the solution The dissolving of keratins in sulphide solutions seems to be a

solution rather than a hydrolysis Constitutionally the keratins are remarkable for they contain large quantities of both cystine and tyrosine (Table I column VI (*a*) and (*b*)) It will be seen later (Chapter V) that the body cannot itself synthesise these two acids and that cystine especially is an essential factor in the daily metabolism of the animal It is difficult to understand therefore why cystine should accumulate in these epidermal organs which play no further part in the cycle of metabolism and on the evidence at present available the arrangement strongly suggests physiological waste The keratins are not attacked by pepsin trypsin or bacterial tryptases Their resistance to decomposition and to digestion by enzymes suggests that there must be special structural linkages in the keratin molecule Stary (1924 1925) finds that under the action of bromine and glacial acetic acid hair is converted into a soluble and digestible form At the same time the carbonyl reactions cease to be given and it is suggested therefore that anhydride rings have been opened to form peptide chains

Biologically the keratins give the animal body mechanical protection and preserve it from bacterial attack Economically they are an important group since wool and silk both come under this heading besides less important materials such as tortoiseshell feathers hair etc

(7) *The Gliadins or Prolamins* —This class of proteins is peculiar to the vegetable kingdom and is found only in the seeds of cereals These proteins are insoluble in water soluble in acid and very readily soluble in dilute alkali They are also soluble in 75 per cent alcohol and this characteristic distinguishes them from all the other proteins Constitutionally they contain large quantities of glutaminic acid and also a high content of proline Their name *prolamin* is derived from this fact (Table II column III) They usually contain cystine and tryptophane but their content in the basic amino acids is low Zein the gliadin of maize contains neither lysine nor tryptophane and the food value of this

cereal is very poor The gliadins have not a high food value if used alone but if used to supplement other proteins which are rich in the units which they lack their food value is considerable The chief value of the cereal seeds is due to the supplementary values of the gliadins and the globulins which are found with them

(8) *The Glutelins*—These are also found only in cereal seeds and always in association with the prolamins They contain a large quantity of glutaminic acid (Table II column IV) They are insoluble in water and salt solutions and very readily soluble in dilute alkalis Like the gliadins their food value when used alone is low Their most important characteristic is their power of making a gas retaining dough with water The successful making of bread is due to the properties of glutelin which is present in large quantities in wheat flour This special property will be discussed later

Group II The Conjugated Proteins

(9) *The phosphoproteins* are animal proteins the most important being caseinogen from milk and vitellin from egg yolk They are products of cell activity and their biological function is to provide food material for the developing embryo or the growing young This function is reflected in their constitution for they contain considerable quantities of the basic amino acids and caseinogen is particularly rich in tryptophane which it will be seen later (Chapter VI) is an essential constituent of mammalian diet The phosphoproteins contain a large quantity of glutaminic acid and have a character more acidic than basic Freshly precipitated caseinogen is iso electric at *p*H 4 6 but dissolves fairly readily at reactions more acid or more alkaline than this Caseinogen which has once been dried undergoes some change that reduces its solubility in acids Ordinary commercial casein is insoluble in water and weak acids and readily soluble in weak alkalis It is precipitated from

alkaline solutions by the addition of a weak acid but on further addition of acid it redissolves The change which casein undergoes on drying is therefore reversible and is not to be confused with the irreversible heat coagulation of the albumins and globulins

The phosphoproteins contain phosphorus which can be released as phosphoric acid by the action of certain enzymes (see pp 77–78) The protein phosphoric acid complex is possibly an ester In milk and egg yolk the phosphoproteins are present in the form of their calcium salts and it is this content of calcium and phosphorus as well as the content of the more important amino acids that makes them such a valuable food for the young growing mammal since recent work has shown the very great importance of the calcium phosphorus ratio in the healthy development of the skeleton The constitution of casein is shown in Table I column VII p 47

Economically the greatest value of the phosphoproteins is their food value but casein which is cheap and readily prepared in a fairly pure condition from milk is also an important technical industrial material and is used for making adhesives and waterproof paints and varnishes

(10) *The hæmoproteins* of which hæmoglobin and hæmocyanin are the chief examples are animal proteins in which the protein is allied to a pigment The hæmoproteins have a respiratory function Hæmoglobin contains iron in its molecule hæmocyanin contains copper Both proteins have been obtained crystalline and in a high state of purity The protein of hæmoglobin is a histone

(11) *The mucoproteins* are bodies in which a protein is associated with a complex carbohydrate radicle which releases a reducing sugar on hydrolysis According to Levene (1925) the associated or prosthetic group is always built up from acetic acid glucuronic acid sulphuric acid and an aminohexose all in unimolecular proportions The mucoproteins are esters of a protein and this complex conjugated sulphuric acid In the

absence of any knowledge on the nature of the protein group Levene classifies the mucoproteins according to the constitution of the aminohexoses into two groups (*a*) The *mucins* in which the associated group is mucoitin sulphuric acid containing glucosamine These are slimy proteins with a lubricating function The mucins secreted by the salivary glands and mucous cells of the alimentary canal may be regarded as typical examples The mucins of the vitreous humour ovomucoid and serum mucoid also belong here (*b*) The *mucoids* in which the associated group is chondroitin sulphuric acid containing galactosamine These are somewhat viscous proteins with a supporting and protective function very widely distributed through the body particularly in tendons and connective tissue

(12) *The Nucleoproteins* —These bodies are of great biological importance since they form the most important protein constituent of the cell nucleus without which cell life and cell activity is impossible They consist of in association between a protein and nucleic acid The nucleoproteins occur in both the animal and vegetable kingdoms They are difficult to isolate and have no special industrial importance

Group III The Derived Proteins

The members of this group may be regarded as forming the series of stages which must be passed through either in breaking down or building up the large protein molecule The group as a whole shows the gradual loss of colloidal characters with increasing degradation of the protein The definitions of the sub groups given in the table of classification above are based on the method of separation employed by Borsook and Wasteneys (1923)

The *metaproteins proteoses* and *peptones* are like the proteins bodies of unknown structure Metaproteins and proteoses are only known as hydrolytic products of proteins Peptone like bodies have been found occurring

naturally in plant and animal tissues Peptones therefore may be regarded as formed both during analysis and synthesis of proteins The peptone sub group shows the disappearance of the colloidal properties of the proteins

The peptides are bodies of known constitution and are formed by the condensation of two or more amino acids by means of a peptide link They have been isolated from among the products of protein hydrolysis usually as dipep tides though Abderhalden (1911) and Fischer and Abder halden (1907) have isolated a tri peptide and a tetra peptide from silk Peptides have also been found occurring naturally in plant and animal tissues Carnosine a di peptide present in fresh meat is β alanyl histidine The most interesting natural peptide is glutathione (glutamyl cysteine) isolated by Hopkins (1921) and shown by him to have an important respiratory function in the living cell and a remarkable power of promoting the oxidation of proteins *in vitro* (1925) Peptides have been synthesised from free amino acids in varying degrees of complexity up to nineteen units The majority of the peptides dialyse through parchment a few of the higher synthetic polypeptides have colloidal properties

Diketopipera zines and *peptic a hydrolysate* condensed from three or four amino acids have also been isolated from the products of protein hydrolysis The evidence of their pre existence in the protein molecule is dealt with in Chapter V

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CHAPTER IV

THE IDENTIFICATION OF PROTEINS AND THE STRUCTURE OF THE PROTEIN MOLECULE

Identification by Nitrogen Distribution—Identification by Racemisation —Structural Pattern and Biological Reactions—Free Amino Nitrogen and Amide Nitrogen—Glucoside Structure in Proteins

Identification by Nitrogen Distribution

An unknown protein can generally be readily assigned to its characteristic class by means of the usual tests *e g* colour reactions solubilities precipitation tests described in Chapter I It is a much more difficult problem to decide whether two proteins in the same class are identical or only closely allied In some cases it is possible to study some special physico chemical property and to compare the quantitative data obtained for the different proteins under investigation for instance Stedman and Stedman (1925) have examined the oxygen dissociation curves of the hæmo cyanins from two species of lobster and two species of crab The identity of the four curves obtained strongly suggests the identity of the four proteins in question though in view of the fact that the hæmoglobins of different mammalian species have been shown to differ in their iron/sulphur ratio it cannot be regarded as proof The properties of the respiratory proteins are highly specialised and belong rather to the prosthetic group than to the protein itself With the other proteins evidence of identity has to be sought by less direct means For instance how is the question to be settled as to whether the two globulins in mammalian serum are the same substance in different states or are two different though closely similar proteins ? A complete hydrolysis with the determination of all the separate units could be attempted but even if carried out with the most scrupulous care would not give results sufficiently accurate to be con

vincing An examination of the nitrogen distribution by the method of Van Slyke might be expected to give more reliable figures Hartley (1914) used Van Slyke's method to compare the proteins of serum His analytical results are shown in Table III He showed definitely that the two globulins of serum the euglobulin and pseudoglobulin (see Chapter II) although differing in their properties are constitutionally the same protein whereas the albumin was differentiated by its higher content of lysine and cystine Hartley's work disproved a suggestion that had previously been made that globulin might arise from albumin by a process of denaturation (see Chapter XIII) but confirmed the idea that euglobulin was a derivative of pseudoglobulin Van Slyke's method has also been used by Crowther and Raistrick (1916) in an investigation of the relations of the globulins of serum to the globulins in milk and colostrum the first milk produced after the birth of the young Milk and colostrum are secreted by the mammary gland of mammals and contain three proteins namely caseinogen lactalbumin and lactoglobulin The two latter are closely similar in general properties to the albumin and globulin occurring in the serum Caseinogen which occurs nowhere in the body except in the mammary gland must obviously be synthesised there but the possibility exists that the albumin and globulin present in the milk may be derived by filtration from the blood plasma Crowther and Raistrick (1916) determined the nitrogen distribution in the protein of cow's milk by Van Slyke's method and compared the figures which they obtained with the nitrogen distribution of the globulins from cow's serum Their experimental results are given in Table III on p 61

It is obvious from these figures that the nitrogen distribution in the different proteins shows certain significant differences Casein is definitely marked off from the other proteins by its higher content of amide nitrogen humin nitrogen and non amino nitrogen and its lower content of mono amino nitrogen Between lactalbumin and lactoglobulin significant differences appear in the nitrogen

TABLE III

		Amd N	Hn N	Cyt N	Ag N	Htd N	Ly N	Mm N	Nm N	Ttl N
Milk proteins (Crowther and Raistrick)	Caseinogen	10 43	3 43	1 95	7 ol	4 24	7 86	55 04	9 51	99 97
	Lactalbumin (milk)	7 72	1 65	2 34	7 27	4 20	13 08	60 91	2 03	99 20
	(colostrum)	8 07	1 93	2 07	7 75	4 60	12 19	59 13	3 06	98 80
	Lactoglobulin (milk)	8 10	2 16	1 87	10 94	4 24	9 16	61 81	1 19	99 46
	(colostrum)	18	2 1C	1 92	10 68	3 74	8 15	63 83	1 09	98 74
Serum proteins (Crowther and Raistrick)	Euglobulin	7 10	2 20	2 02	10 81	3 78	8 13	63 77	0 87	98 68
	Pseudoglobulin	7 26	2 12	1 82	10 56	3 70	8 16	63 90	1 30	98 81
Serum proteins (Hartley)	Albumin	7 0o	1 60	2 75	11 0o	4 40	13 55	56 55	2 15	99 20
	Euglobulin	9 3	2 0	2 0	11 6	3 8	9 2	57 9	2 8	98 6
	Pseudoglobulin	7 o	1 9	1 9	10 8	4 8	9 6	61 7	1 6	99 8

figures for the arginine lysine and non amino fractions The figures for the lactalbumin of both milk and colostrum are the same and the figures for the globulins whether derived from milk colostrum or serum are identical within the small experimental error of the method

When however the figures obtained for lactalbumin are compared with Hartley's figures for serum albumin it can be seen that these two proteins differ from each other considerably almost as much as each one differs from globulin

Crowther and Raistrick concluded from their results that the globulin of milk is the same protein as the globulin of serum which can exist in two forms euglobulin and pseudo globulin They therefore considered that lactoglobulin is not synthesised in the mammary gland but filters through to the milk from the blood The first milk the colostrum is very rich in globulin containing as much as 6 12 per cent of this protein whereas less than 0 4 per cent occurs in normal milk It seems therefore that immediately after the birth of the young mammal there is an increased permeability of the walls of the capillaries of the mammary gland which allows a considerable escape of globulin from the blood Lactalbumin however differs so definitely from serum albumin as to suggest an actual synthesis in the mammary gland

Identification by "Racemisation"

The origin of the proteins in milk and the problem of their identity has been further studied by Dudley and Woodman (1915 1918) and by Woodman (1921) Their work was based on an observation of Dakin (1912–13) that in strongly alkaline solutions the specific rotation of a protein drops slowly from its original value to a much lower one at which it remains stationary Dakin called this change racemisation His work will be described in detail later Woodman (1921) found that every protein has a definite rate of racemisation under fixed conditions which can be followed by plotting the curve of specific rotation against time and therefore that the racemisation curves could be used to test

the identity of proteins In Fig 1 are shown Woodman s racemisation curves for the globulin of cow s colostrum and cow s serum in 2 per cent solution at 37 curve A showing the rate of loss in N/4 sodium hydroxide and curve B in N/2

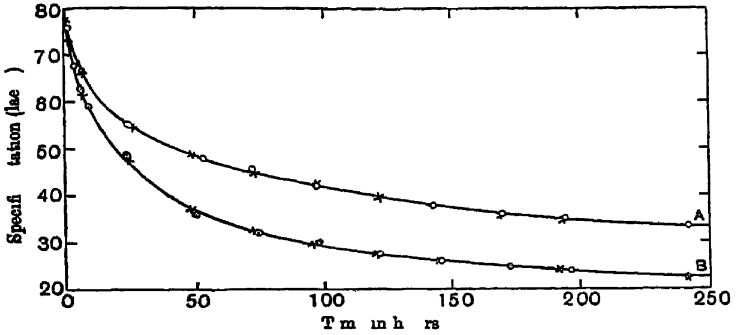


FIG 1 —Racemisation of cow colostrum pseudoglobul n (×) and cow serum pseudoglobulin (○)
Curve A—2 per cent protein in N/4 NaOH
Curve B—2 per cent pr te n in N/2 NaOH
(From Woodman *B oche n cal Journal* 1921)

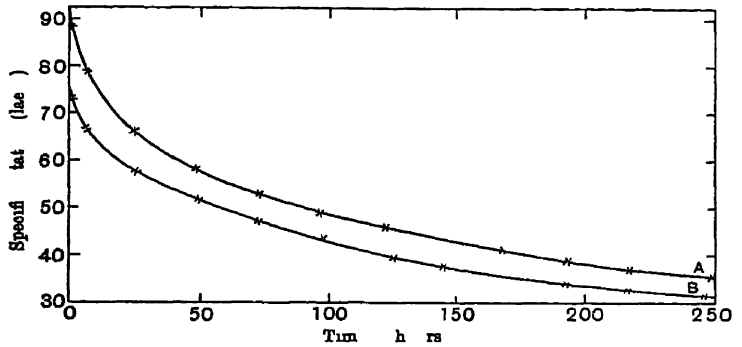


FIG 2 —Racemisation of 2 per cent albumins in N/4 NaOH
Curve A—cow colostrum albumin
Curve B—ox serum albumin
(From Woodman *Biochemical Journal* 1921)

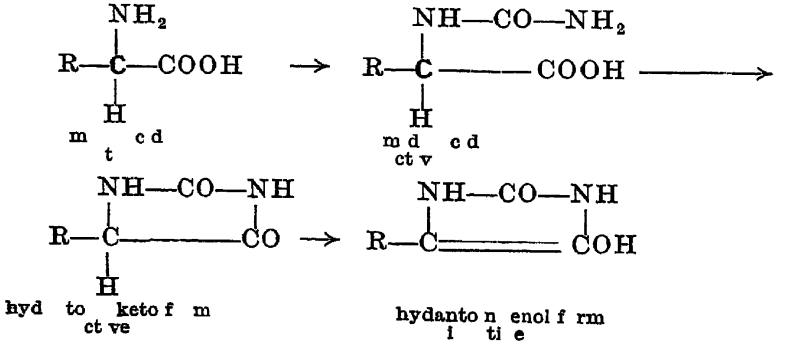
It is obvious that the two proteins show exactly the same behaviour under the same conditions and may therefore be considered to be identical In Fig 2 are shown the curves for colostrum albumin and serum albumin The two curves start at different initial points and follow different though parallel courses The two proteins therefore though similar cannot

be identical These experiments of Woodman are strong evidence of a synthesis of albumin in the mammary gland They confirm the conclusions of Crowther and Raistrick that the globulin of milk is derived by filtration from the serum

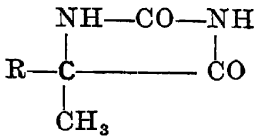
Structural Pattern and Biological Reactions

Dakin's observation of the racemisation of proteins in strongly alkaline solution was used by him not only as a test for the identity of proteins but also to obtain evidence on structural pattern in the protein molecule It is obvious that where so many units are built into the molecule the final structural pattern in two proteins may differ even though the number and kinds of units in them may be the same and moreover that their properties may be affected by the difference in structure Dakin obtained evidence on molecular structure by an acid hydrolysis of normal and racemised proteins

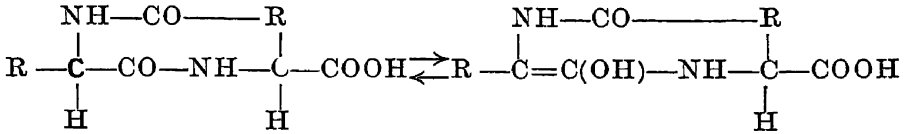
Amino acids released from an acid hydrolysis of a protein are with the obvious exception of glycine optically active If however the protein has previously been allowed to stand in contact with an alkali hydrolysis gives different results *i e* whilst some of the amino acids released have their normal optical activity others appear in an inactive form This change is called racemisation an unsuitable term since the loss of optical activity comes not from a mixture of *d* and *l* forms but from a conversion into an inactive form Dakin was led to the study of the racemisation of proteins by a similar change in the hydantoins —



The change rom the active keto to the inactive enol form is spontaneous in aqueous solutions of hydantoins If the hydantoin is α methylated the change to the enol form cannot take place



and the product is permanently optically active Dakin therefore suggested that under the action of an alkali at low temperature the following similar change might occur in proteins



This keto enol transformation cannot take place in an amino acid with a free carboxylic group *i e* one which may be supposed to occupy a terminal position in a chain but only in one completely built into the molecular structure It follows therefore that after an acid hydrolysis of a race mised protein with splitting of the peptide link the amino acids from terminal position should appear in the normal active form the others in the inactive form

Dakin points out that the action of dilute all ali at low temperatures (37 C) although accompanied by some hydrolysis is quite unlil e that of concentrated all ali at high temperatures For example he found that 10 per cent gelatin in 1 7 per cent sodium hydroxide at 37 C had an initial rotation of — 13 5 which fell for fifteen days to — 2 32 after which it was stationary Acid hydrolysis at this stage led to the separation of active and inactive amino acids as shown in table at top of p 66

The balance of active and inactive acids therefore gives some evidence of the arrangement of the units in the mole cule Dakin suggested that the active acids pre exist in the

Alkaline Racemisation of Gelatin followed by Acid Hydrolysis

Optically	Inverted
Lysine	Leucine
Glutaminic acid	Phenylalanine
Some proline	Histidine
Some alanine	Arginine
	Some proline (largely racemised during separation)
	Some alanine
	Glycine

molecule at the ends of branched chains are rapidly split off and escape racemisation Groupings further into the molecule so to speak undergo racemisation before they are set free by hydrolysis

Dakin and Dale (1919) used the racemisation method as a crucial test as to the identity of the crystalline albumins of hen and duck eggs In chemical constitution these two proteins are closely allied but biologically they have different reactions A guinea pig sensitised by a preliminary injection of 1 mg of hen albumin died in four minutes after a second dose of 0 0⁄ mg of hen albumin (anaphyllactic shock) whilst an animal which had been similarly sensitised with hen albumin only showed slight symptoms when injected with 0 15 mg of duck albumin Conversely a guinea pig sensitised by a preliminary injection of 1 mg of duck albumin died of anaphylaxis on receiving a second dose of 0 05 mg though a similarly sensitised animal showed no symptoms on receiving 0 05 mg of hen albumin Racemisation and hydrolysis of the two proteins showed that though constitutionally allied different amino acids in the two cases became inactivated during racemisation definite differences occurring in the optical properties of the leucine aspartic acid and histidine from the two albumins These results of Dakin and Dale show that the individuality of a protein depends not only on the number and kind of units it contains but also on molecular pattern Dakin and Dudley (1915) made acid hydrolyses of casein and gelatin and

showed that the position of these acids was different in these two proteins In casein only the proline some leucine and some valine escaped inactivation while in the gelatin proline lysine glutaminic acid and some alanine were recovered in the optically active form Dakin and Dudley showed that racemisation renders the proteins completely resistant to the action of the proteolytic enzymes a fact which is in harmony with the suggestion that a transformation occurs at the peptide link

Free Amino Nitrogen and Amide Nitrogen

A little more disconnected evidence exists on the placing of the units in the protein molecule Kossel (1902) and Kossel and Cameron (1912) showed that the acid binding power of the protamines was exactly equivalent to the amino groups of the guanidin nucleus of the arginine present They considered these must be free and arginine therefore is bound into the molecule by one amino group only The amino group of guanidin does not give off gaseous nitrogen under the action of nitrous acid (1911) Van Slyke and Birchard (1913) made the interesting observation that in an intact protein the gaseous nitrogen given off under these conditions was always equal to half the lysine nitrogen This leads to the supposition that lysine also has always one but never both of its basic groupings bound into internal linkages The work of Dudley and Dakin mentioned above suggests however that the acid grouping of lysine may in some proteins be bound and in others free

Osborne Leavenworth and Brautlecht (1908) showed that in the plant proteins the percentage of dicarboxylic acids present was proportional to the percentage of ammonia released on hydrolysis suggesting that in these acids one carboxylic grouping is present as an acid amide in a terminal position This generalisation does not seem to hold for the animal proteins and Dakin and Dudley's results on

racemised casein and gelatin suggest that the position of the glutaminic acid is different in these two proteins

Glucoside Structure in Proteins

In the earlier history of protein chemistry there was much discussion as to whether proteins such as ovalbumin serum albumin etc which even when prepared by crystallisation still yielded a reducing sugar on hydrolysis contained a carbohydrate group (glucosamine) combined in the protein molecule Langstein (1922) finds as much as 0 5—1 per cent of reducing substances even in carefully purified globulin It was thought at one time that proteins had a glucoside structure but it still seems possible that the ubiquitous presence of the mucins is responsible for the carbohydrate reaction even in preparations supposed to be of a high degree of purity The problem is very lucidly reviewed by Levene in his book on The Hexosamines and the Mucoproteins (1925)

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CHAPTER V

THE PROTEOLYTIC ENZYMES AND THE LINKAGES IN THE PROTEIN MOLECULE

The Proteolytic Enzymes Pepsin Trypsin Erepsin—Hydrolysis of the Peptide Link—Protein Synthesis—Types of Linkage in the Molecule—The Position of Phosphorus—The Position of Sulphur

The Proteolytic Enzymes

The proteolytic enzymes are those which catalyse the hydrolytic breakdown of proteins in aqueous solutions They are secreted by bacteria moulds and all animal and vegetable tissues and are known by the general name of tryptases The tryptases of tissues and the bacterial tryptases are probably always mixtures of enzymes The former have an optimum activity at *p*H 4 5—5 0 the latter in neutral or faintly alkaline media The proteolytic enzymes which have been most studied however are those produced by the digestive system of vertebrate animals These enzymes are probably each of them a distinct chemical species They are among the earliest known and still retain the early nomenclature with the in termination

The proteolytic enzymes of the digestive system of vertebrates are *Pepsin* secreted in the stomach *trypsin* secreted by the pancreas and *erepsin* secreted by the small intestine The optimum reaction for peptic digestion is *p*H 2 0 for tryptic 8 0 and for ereptic 7 8

Pepsin—Pepsin can hydrolyse all classes of proteins except keratins It readily attacks most albumins and globulins collagens reticulin and the vegetable proteins Nevertheless Frankel (1916) found that pepsin is unable to hydrolyse more than about 20 per cent of the peptide linkages present in a

protein (see Figs 3 and 4) Pepsin has no action on peptones or peptides whether naturally occurring or synthetic

Trypsin—Trypsin cannot attack directly collagens reticulin or keratins It rapidly hydrolyses elastin It can act directly on casein (the stomach of the young infant does not secrete pepsin) and some of the albumins and globulins Trypsin acting directly on a protein substrate hydrolyses approximately 60 per cent of the peptide linkages (Irvine 1916) Trypsin acting on a protein after peptic treatment will hydrolyse approximately 70 per cent of the peptide links (see Figs 3 and 4) The action of the pepsin has therefore been to make a certain number of peptide linkages susceptible to tryptic action Many proteins which resist the action of trypsin can by boiling by peptic digestion by slight acid or alkaline hydrolysis be made hydrolysable by trypsin Collagen for instance after having been boiled is hydrolysed by trypsin Gelatin derived from collagen by mild acid hydrolysis is very rapidly hydrolysed by trypsin Trypsin can hydrolyse peptones and many but not all of the synthetic polypeptides Certain of the peptide links therefore can resist tryptic action Trypsin hydrolyses glycyl *l* tyrosine releasing tyrosine which appears as a crystalline precipitate as the hydrolysis proceeds This reaction is used to distinguish trypsin from pepsin which cannot hydrolyse the peptide Silk peptone a mixture of peptides containing a large percentage of glycyl *l* tyrosine is usually used for the test

Erepsin—Erepsin strongly resembles trypsin but has an even greater hydrolytic power A tryptic digest always gives a biuret reaction while an ereptic digest ultimately fails to give this reaction Erepsin has no direct action on the majority of naturally occurring proteins but after either peptic digestion or peptic and tryptic digestion erepsin can carry the hydrolysis of the peptide linkage to within about 90 per cent of completion

In Figs 3 and 4 are shown two sets of experimental

curves taken from Frankel s paper The curves illustrate the percentage of the total amino N appearing as free amino nitrogen plotted against time The first set (Fig 3) shows the progressive hydrolysis of gelatin measured by the

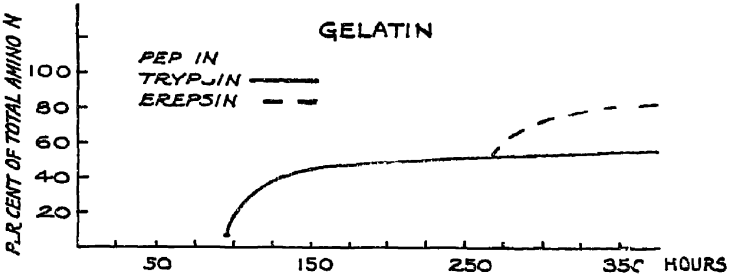


FIG 3 —Hydrolysis of gelatin by pepsin trypsin and erepsin (From Fianlel *Journal of Biological Chemistry* 1916)

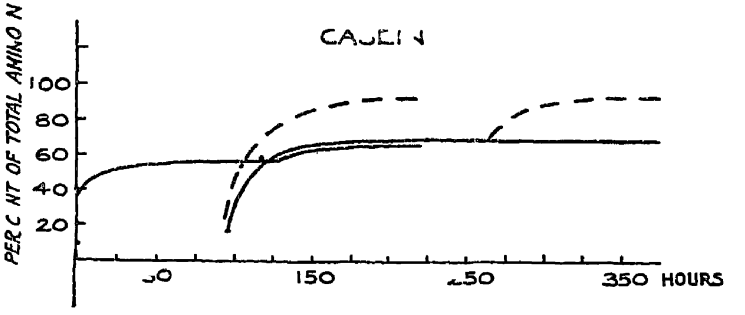


FIG 4 —Hydrolysis of casein by pepsin (dotted line) trypsin (continuous line) pepsin and trypsin pepsin and erepsin (dot dash) pepsin and trypsin and erepsin
(From Frankel *Journal of Biological Chemistry* 1916)

total amino nitrogen present in the digestion fluid during the action of pepsin (1) pepsin followed by trypsin (2) pepsin followed by trypsin followed by erepsin (3) the other set (Fig 4) shows the digestion of casein by pepsin (1) trypsin (2) pepsin followed by trypsin (3) pepsin followed by erepsin (4) pepsin followed by trypsin followed by erepsin (5) It is obvious that pepsin has some action distinct from that of the two other enzymes and it seems not unreasonable to suppose that the action of trypsin and erepsin is practically limited to a hydrolysis of the peptide

link and that in the cases where these do not initiate the hydrolysis of a protein the peptide link has been protected by steric hindrance within the molecule from which however it can as it were be released by the action of pepsin Erepsin cannot open the diketopiperazine ring (Levene and Pfaltz 1925)

Hydrolysis of the Peptide Linkage

The hydrolysis of the peptide link has recently been studied by Levene Simms and Pfaltz (1924) and by Levene and Simms (1924) They studied the hydrolysis of the four peptides formed by the four possible combinations of glycine and sarcosine (methylglycine $CH_2(NHCH_3)$ $COOH$) They give the following experimental figures for the relative rates of hydrolysis (K) of the four dipeptides together with the ionisation (dissociation) constants of the two amino acids and the four dipeptides when functioning as an acid (K_a) or a base (K_b) respectively

Substance	Isoelectric point	$K_a \times 10$	$K_b \times 10$	Relative rates of hydrolysis N/100
Glycine	5 98	4 68	7 24	—
Sarcosine	6 12	5 89	17 0	—
Glycyl glycine	5 59	0 759	0 19	100
Sarcosyl glycine	5 80	0 794	0 273	64
Glycyl sarcosine	5 68	1 48	0 576	57
Sarcosyl sarcosine	5 98	1 38	2 09	15

They find that three reactions are involved in an acid hydrolysis —

$$\text{Anhydride} \underset{(3)}{\overset{(2)}{\rightleftharpoons}} \text{Peptide} \xrightarrow{(1)} 2 \text{ amino acid molecules}$$

and they find that the tendency towards formation (or resistance to hydrolysis) of ring compounds (anhydrides) of the di peptides is proportional to the product of the dissocia

tion constants of the groups involved in the linkage The same generalisation holds with ereptic action except that the opening up of the anhydrides is not promoted so readily This work of Levene and his co workers throws new light both on the possible mechanism of the action of the different proteolytic enzymes and on the structure of the protein molecule If the hydrolysis and synthesis of proteins can be represented generally by the scheme —

$$\text{Anhydride} \underset{(3)}{\overset{(2)}{\rightleftharpoons}} \text{polypeptide} \xrightarrow{(1)} \text{amino acids}$$

the following possibilities suggest themselves —

That acids catalyse reactions (1) and (3) reaction (2) only to a very slight extent

That erepsin (and probably trypsin) catalyses reaction (1) reaction (2) rather more than acid reaction (3) not at all

That pepsin has no catalytic effect on (1) but a marked effect on (2) and (3)

Protein Synthesis

The recent work of Borsook and Wasteneys (1923) has an interesting bearing on this possibility They found that at a *p*H of 1 7 pepsin hydrolyses egg albumin with the production of peptones a stage of protein degradation which is not precipitated by trichloracetic acid and which gives a rose pink colour in the biuret reaction If however the reaction of a concentrated digestion mixture is adjusted to *p*H 4 in the presence of pepsin a precipitate forms in about three days at 37 which on examination shows all the properties of a true protein It is soluble gives a mauve biuret reaction and is completely precipitated by trichloracetic acid and is hydrolysed by pepsin at *p*H 1 7 It seems therefore justifiable to assume that in solutions of protein at *p*H 1 7 pepsin catalyses principally reaction (3) while in solutions of peptones at *p*H 4 its chief effect is a catalysis of reaction (2)

Borsook and Wasteneys have shown therefore that by controlling conditions pepsin can synthesise as well as hydrolyse Abderhalden and Fodor (1922) have also obtained evidence of the synthetic action of pepsin Wasteneys and Borsook have been unable to obtain a synthetic action by pepsin in the hydrolytic products of gelatin They have also shown (1925) that trypsin at *p*H 5 7 has a definite synthetic action on a peptic digestion mixture Trypsin at *p*H 5 7 therefore catalyses reaction (2) At 8 its chief action is to catalyse reaction (1) There is also slight catalysis of reaction (1) even at *p*H 5 7 with the production of free amino acids occurring simultaneously with the synthesis This observation confirms some earlier work by Henriques and Gjaldbak (1912) The work of Wasteneys and Borsook has an important bearing on the problem of protein synthesis in the cell Tissue enzymes of a tryptic nature and more rarely of a peptic nature have been found in all animal and plant tissues Are these to be regarded as the means by which protein is synthesised in the living cell ?

The accepted theory of enzyme action is that the enzymes are organic catalysts and as such should accelerate the attainment of an equilibrium condition from either side *i e* the proteolytic enzymes should have not only a hydrolytic but also a synthetic action in the body The work of Wasteneys and Borsook is evidence in support of this idea by altering conditions the equilibrium

$$\text{proteins} \rightleftharpoons \text{peptones}$$

is shifted and pepsin catalyses the attainment of equilibrium from either side It follows from this that the activities of enzymes present in living cells should be controlled by physico chemical conditions The release of autolytic activity following the increased acidity due to post mortem changes in cells is evidence that the activity of the cell enzymes is under the control of external physical conditions Wasteneys and Borsook consider that in their experiments

only one enzyme is active in stimulating either hydrolysis or synthesis Maignon (1924) however believes that special synthetic enzymes are to be found in the tissues The tissue tryptases like pancreatic trypsin hydrolyse synthetic polypeptides but their point of attack is frequently a different one

Further work on the nature of the mechanism by which acids alkalis and the proteolytic enzymes attack proteins is recorded by Northrop (1921/2) In the early stages of the hydrolysis of gelatin he considers that alkali trypsin and pepsin all hydrolyse by similar methods but that acid hydrolysis follows a different course For further work on this subject reference should be made to Northrop's original papers (1922/3 1923/4)

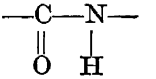
Types of Linkage in the Molecule

But to return to the problem of the linkages in the protein molecule—the existence of the peptide link has been recognised since the time of Fischer and the strength of the link has now been shown by Levene and his co workers to depend on the ionisation constants of the amino and carboxylic groups which take part in its formation The existence of anhydride rings in the protein molecule and the possibility of the formation of internal salts has also long been recognised collagen for instance has for many years been recognised as an anhydride of gelatin It seems very probable that the strength of the anhydride ring (diketopiperazine) also will be found to depend on the nature of the groups which form it Many peptide linkages resist the action of trypsin and possibly many anhydrides resist the action of pepsin—the keratins for instance are remarkable for the power of resisting enzymes yet hydrolyse to amino acid mixtures under the action both of acids and alkalis Boiling in water seems an efficient method of opening anhydride rings After boiling with water collagen is hydrolysed by trypsin A few of the plant globulins when prepared by dialysis are remarkably resistant to the action of

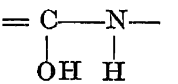
the digestive enzymes Waterman and Breeze Jones (1921) showed that the globulins (stizolobin) from both the Chinese and the Georgia velvet bean when prepared by dialysis were only digested *in vitro* to the extent of about 30 per cent by pepsin followed by trypsin When the dialysed proteins were boiled or when the globulins were prepared by a coagulation method the digestibility rose to nearly 60 per cent and it seems possible therefore that both these processes lead to an opening up of anhydride rings (see also Chapter XIII) The probable existence of diketopiperazine rings in the protein molecule has received additional support from the recent work of Goldschmidt and Stiegerwald (1925) who showed that in the degradation of proteins by hypobromites the course of the reaction was similar to that which occurs with diketopiperazines The possibility of the existence of pyrazine rings that is 2 5 diketopiperazines in the enol form has been discussed by Karrer Grunacher and Schlosser (1923) but their presence in the molecule has not been proved experimentally

There are therefore the following linkages the existence of which in the molecule may be regarded as certain

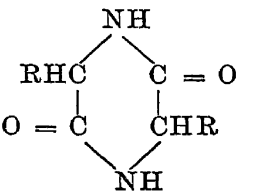
1 *The peptide linkage*



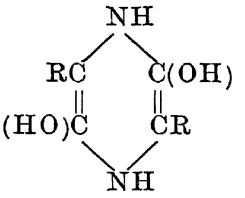
which may be possibly transformed under the action of alkalis to an enolic form resistant to tryptic action



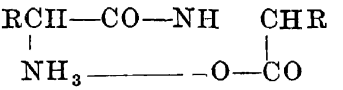
2 *The* 2 5 *diketopiperazine linkage*



the existence of which in the intact protein has been demonstrated by Abderhalden and Komm (1924) Theoretically this linkage could also exist in the enol form as a pyrazin ring —



Would this form also be resistant to enzyme hydrolysis ? From the structure of the molecule the possibility exists and there may also occur not only the formation of internal anhydrides but also of internal salts of the substituted



ammonium types Such bodies if they exist would be hydrolysed in solution

Many changes which proteins undergo without actual degradation of the molecule such as denaturation conversion into metaprotein etc are probably accompanied by an opening or new formation of rings in the molecule

The Position of Phosphorus

The existence of another type of linkage in the molecule of casein has recently been suggested by the work of Rimington and Kay (1925) They find that trypsin rapidly splits off the phosphorus from casein in the form of an organic compound which is further hydrolysed slowly to yield inorganic phosphates The organic phosphorus complex is very rapidly hydrolysed by Robison s bone enzyme (1924) which has no direct action on the casein itself Phosphorus therefore is bound into the casein molecule by two linkages—the peptide link and the linkage of another type split by the bone enzyme which liberates phosphoric acid from its esters

The Position of Sulphur

The linkages by which sulphur is bound into the protein molecule are uncertain It is partly present in cystine groupings from which it is released under the action of strong alkalis as a sulphide Osborne (1902) gives a number of analytical figures for total sulphur and sulphide sulphur in proteins (see pp 46–48 Tables I and II) and suggests that the non sulphide sulphur is present as sulphydryl in cysteine groupings Mueller (1923) has reported a new amino acid containing sulphur in yet another type of combination Intact proteins do not generally have either a reactive sulphide or sulphydryl group though these may appear after denaturation (Harris 1923 Walker 1925) There is no direct connection between the sulphide group and the peptide linkage in proteins (Stary 1924 1925)

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CHAPTER VI

THE CHEMISTRY OF PROTEIN FOODS

Protein and Nitrogen Loss—Amino Acid Synthesis—Protein Synthesis—Special Units Tryptophane and Lysine Cystine Histidine and Arginin Proline Tyrosine and Phenylalanine—Digestibility of Proteins—Biological Value of the Proteins

Protein and Nitrogen Loss

THE tendency of the animal organism to use protein as material out of which to manufacture a number of substances of varying physiological function leads to a constant loss of protein from the system Thus in the mammal the wearing away of the keratinous outer surface of the epithelium the growth of the hair and the secretion of mucus in the alimentary canal all lead to a steady loss of protein and hence of nitrogen Besides these obvious sources of loss the metabolic cycle of the body also leads to a further loss of nitrogen If an examination is made of the nitrogen in the urine it is found that even under conditions where no nitrogen is supplied in the food there is an excretion of nitrogen by the body of about 2 5 grams per day for a man of average weight that is about 3 5 milligrams to every kilo of body weight The distribution of the urinary nitrogen is shown in the following table taken from a paper by Folin (1905)

	A g d t Mlligm p kl f bd w glt (24)	L w P t D t Mlligm p kl f bdy w ht (4 h)
Urea	14 0	2 2
NH_3	0 5	0 4
Creatinine	0 57	0 60
Uric Acid	0 23	0 09
Undetermined	0 7	0 27

These figures show very clearly that while the output of urea and uric acid depends on the nature of the food supply there is a steady loss of nitrogen in the form of creatinine under all circumstances (see also Robison 1922)

Both protein and nitrogen must therefore be restored to the body and the question arises as to whether protein as such is necessary in the diet whether amino acids are necessary or whether the animal can synthesise both amino acids and proteins from simpler compounds It will be convenient to consider first the evidence of amino acid synthesis The direct evidence available on this point is somewhat scanty but quite clearly defined

Amino Acid Synthesis

Embden and Smitz (1910–12) showed that if the surviving liver of a dog were perfused with a solution containing the ketonic acid corresponding to tyrosine phenyl alanine or alanine then the corresponding α amino acid appeared in the perfusion fluid Knoop and Kertess (1911) showed that amino acid synthesis is not a peculiar property of the abnormal conditions of the isolated liver but can be affected by a normal animal To demonstrate this they had to administer some substance foreign to the normal course of metabolism since material such as hydroxy or keto acids corresponding to any of the ordinary amino acids would be lost sight of in the normal cycle of metabolism They therefore chose the sodium salt of β phenyl α hydroxy butyric acid and injected it under the skin of a dog β phenyl α amino butyric acid an amino acid not used by the body for protein synthesis was recovered from the urine

The animal body can therefore convert a hydroxy acid to an amino acid and the work of Underhill and Goldschmidt (1913) and of Abderhalden (1915) showing that the ingestion of organic ammonium salts and urea reduces the loss of body nitrogen which occurs on a purely carbohydrate diet sug

gests that the nitrogen required for this synthesis can be obtained from these simple types of compounds The power of synthesising an amino acid from the corresponding hydroxy acids seems however to be limited in extent McGinty Lewel and Marvel (1924) showed that the growing rat is unable to convert α hydroxy ε amino caproic acid into lysine Direct evidence therefore shows that the animal has a limited capacity of synthesising amino acids but throws no light on its power of synthesising the hydroxy acids corresponding to the more complex amino acids

The *role* of protein in diet may now be considered An animal needs food to supply both structural material and energy Numberless experiments on animal feeding have shown that for the maintenance of health in the mammal a mixed diet containing proteins fats carbohydrates mineral salts and water is necessary The importance of the accessory factors of unknown constitution the vitamins is also recognised Nitrogen can be adequately supplied purely in the form of protein and thus the problems of nitrogen supply and protein function become two aspects of one question

Protein Synthesis

It was recognised long since by Loewi (1902) that for protein synthesis in the body *i e* for growth it was not necessary to supply intact protein in the food This has been confirmed by many later experiments

Abderhalden and Rona (1904) fed mice on casein and sugar and on a series of casein digests prepared in different ways They found that a diet of casein digested by trypsin and supplemented with sugar gave as good growth as the untreated casein and sugar diet When pepsin was used instead of trypsin to digest the casein the results were not so satisfactory and finally when sulphuric acid was used to digest the casein the animals rapidly lost weight and died in a few days The unsatisfactory results obtained with the

acid digest were difficult to explain at that stage but it was later shown by Willcock and Hopkins (1907) that the trouble was due to the destruction of the tryptophane of the casein by the boiling acid

Ackroyd and Hopkins (1916) found that young rats grew well and kept healthy on a diet consisting of casein (or casein plus lactalbumin) completely hydrolysed by boiling with 25 per cent sulphuric acid *if* the following additions were made to the diet 2 per cent of tryptophane 0 5 to 1 per cent of cystine and fats starch sugar salt in generous quantity Proteins as such therefore are not essential in the diet As long as enough fibrous material (roughage) is present to provide the necessary stimulus for the peristaltic movements of the intestine the nitrogen required can be supplied entirely in the form of free amino acids

It is obvious from what has been said in the previous chapter that the order of the proteolytic enzymes in the alimentary canal is the one which will ensure the greatest possible degree of hydrolysis of any proteins eaten in the food The amino acids set free in this way and possibly a few of the simpler polypeptides can diffuse from the lumen of the gut into the capillaries of its walls and become available as building material or fuel for the organism

The fate of the amino acids absorbed into the body is interesting and at first a little unexpected for the greater part of the nitrogen which they contain is removed by de amination in the liver and appears as urea in the urine The de aminated residues then follow two separate lines of metabolism—the residues of the straight chain amino acids pass into the fat metabolism of the body and are used as fuel supplies just as fats are used the residues containing a closed ring (unless required for certain specific purposes mentioned later) undergo splitting of the ring and in this condition pass into the cycle of the carbohydrate metabolism *i e* they also go to supply energy At first sight therefore it would seem as if the bulk of the protein consumed in an

ordinary mixed meal should be replaceable by fat and carbohydrate only enough protein being supplied to make good the actual daily loss of nitrogen by the body This however is not the case the explanation being that not only nitrogen itself but also certain of the amino acid units are required by the body not as a source of energy but as structural material Most of the amino acids found in the proteins of the tissues can be synthesised by the body but a certain few cannot and have therefore to be supplied ready made in the diet These special individuals are tryptophane lysine cystine histidine and possibly arginine proline tyrosine and phenylalanine The evidence for this will now be given In all experiments made to determine the influence of one special factor in an animal's diet it is of course necessary to ensure a plentiful supply of all other essentials and to be sure that the calorific content is adequate

Special Units

Tryptophane and Lysine—Willcock and Hopkins (1907) showed that mice would not live on a diet in which the only protein given was zein the gliadin of maize Zein contains neither tryptophane nor lysine (see p 48 Table II column III) and it was found that by simply adding tryptophane to the diet of the mice they remained healthy for a very much longer time By adding tryptophane and lysine the experimental animals could not be distinguished from the controls Tryptophane therefore has some individual *role* to fulfil in metabolism over and above that of supplying a quota of nitrogen to the system and obviously the synthesis of tryptophane or the indole nucleus is beyond the powers possessed by the mouse The individual importance of tryptophane has been confirmed repeatedly and on several different mammalian species Abderhalden (1913) found that it was essential for the maintenance of health and bodily weight in dogs Ackroyd and Hopkins (1916) using digested

casein which contains an adequate supply of lysine (see p 47 Table I column VII) showed that removal of tryptophane from the diet of young growing rats was immediately fol

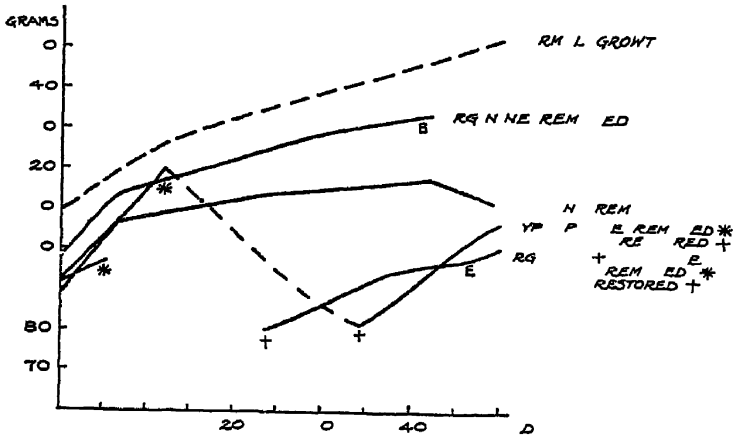


FIG 5 —Influence of tryptophane argin ne a id histid ne on ₅rowth f rats on an amino acid mixtuie (hydrolysed casein)
(From Ackroyd and Hopkins *Biochemical Jour ual* 1916)

lowed by a rapid loss of weight its restoration by renewed growth Their experimental curve (D) is reproduced in Fig 5 Osborne and Mendel (1915) repeated the experiments

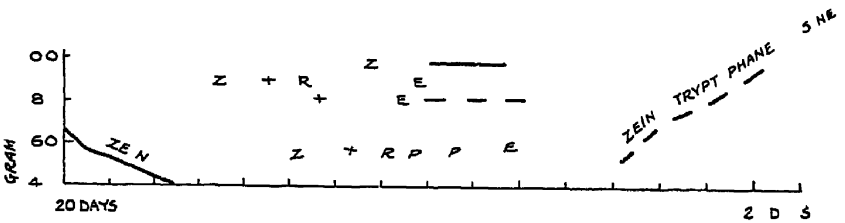


FIG 6 —Influence of tryptophane and lysine on growth of rats on zein a protein in which both groupings are absent
(From Osborne and Mendel *Journal of Biological Chemistry* 1915)

on the effect of zein as the only protein in the diet of youn₅ growing rats They found that on zein the animals rapidly lost weight with the addition of tryptophane to the diet they were able to maintain their weights and keep he ilthy for

long periods but that growth was not resumed with the further addition of *lysine* normal growth was restored Their experimental curve is reproduced in Fig 6 Trypto phane and lysine herefore are essential constituents in diet Osborne and Mendel have also shown the influence of lysine in supplementing edestin a protein which contains trypto phane but is comparatively poor in lysine precursors (see p 48 Table II column II) Their experimental curves are shown in Fig 7 It can be seen from the curves that

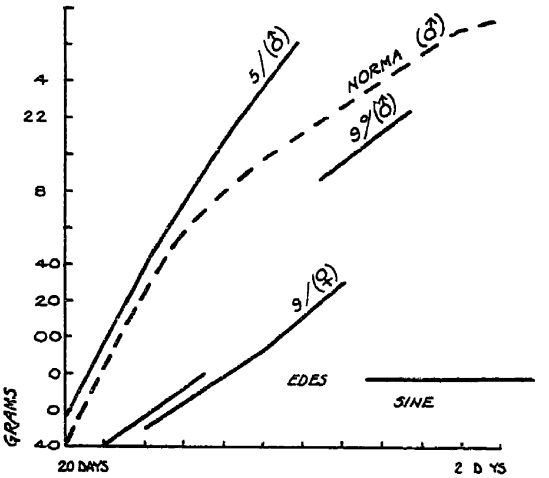


FIG 7 —Influence of ly ne on growth of rats on edest n a prote n wh ch conta ns a small amount of th s grouping
(From Osborne and Mendel *Journal of B olog al Chem stry* 1915)

15 per cent of this protein in the diet promotes normal growth but if the percentage is reduced to 9 growth falls off but can be restored by giving additional lysine The necessity for both tryptophane and lysine in the diet of mice rats and dogs has therefore been established by experiment There is indirect evidence that it is equally important for monkeys and for man The importance of lysine and tryptophane in a diet is graphically illustrated by the occurrence of a condition known as pellagra A very full account of the medical history of this disease is

given by Wilson (1921) the summary of whose conclusions is given below

Pellagra is a disease which only appears among the poorest classes in tropical countries and is nearly always associated with a meagre diet of maize It is characterised by emaciation by a painful rash on the exposed parts of the body by general œdema and by nervous symptoms which may end in definite insanity It can be cured at any rate in its early stages by giving fresh animal protein though in its later stages the damage inflicted on the body seems to be permanent Half the protein of maize consists of zein and the whole maize contains very little tryptophane and lysine both of which are present in fair quantity in all animal proteins The deduction seems justifiable therefore that these two protein units are essential in the diet of man and that their absence leads to ill health During the recent war particularly in Austria under the acute conditions of underfeeding which prevailed there many people developed a condition known as war œdema These cases rapidly disappeared after the arrival of the American Relief Commission and the disease was undoubtedly due more to serious deficiencies in the nature of the war diet than to actual shortage in quantity Chick and Hume (1920) give an account of the experimental results obtained by feeding monkeys for a prolonged period on diets in which the protein supply was a mixture of zein and maize glutelin This mixture contains only traces of tryptophane and lysine In spite of the diet being adequate in all other respects the monkeys lost weight rapidly and developed erythematous rashes on the face and parts of the body and in one case marked œdema The loss in weight ceased in two cases immediately tryptophane was added to the diet and both the œdema and the face rash diminished after the addition of tryptophane and lysine in the form of commercial casein Restoration of weight however only took place after the return to a normal mixed diet

The function of tryptophane in the body is not clear Willcock and Hopkins suggested that the rapid onset of serious symptoms when it is absent from the diet may possibly be due to the loss of a substance absolutely necessary to life—something for instance of an importance equal to that of adrenaline Kendall (1919) isolated thyroxin from the thyroid and stated it to be an iodo derivative of tryptophane His hypothesis has been unfavourably criticised though Hiel s work (1925) on absorption spectra suggests the existence of an indole nucleus in thyroxin Harington (1926) however has shown thyroxin to be a tyrosin derivative

The function of lysine is at present unknown but it occupies a peculiar position in the protein molecule It has already been shown that the free amino groups of all proteins are due to one of the amino groups of lysine and it will be shown later that many of the colloidal propertics of tissue proteins (*e g* colloidal swelling) are due to these free amino groups

Cystine —Cystine is one of the sulphur containing units of proteins Osborne and Mendel (1915) have shown that it is

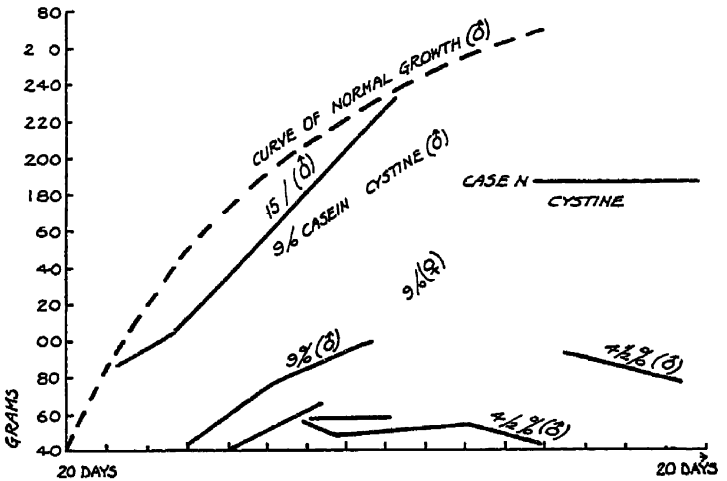
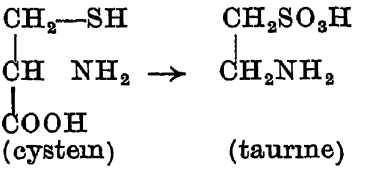


FIG 8 —Influence of cystine on rowth of rats on case n a protein which contains a small amount of th s group ng
(From Osborne and Mendel *Jo ir l of B ological Chem stry* 1915)

essential in the diet of rats Their experimental curves showing the growth of young rats on casein (a protein poor in cystine) and on casein with the addition of cystine are given in Fig 8 It can be seen that with 15 per cent of casein in the diet good growth is obtained but with 9 per cent of protein normal growth is only obtained by giving a supplement of cystine At 4 5 per cent the addition of cystine is unable to restore normal growth Willcock and Hopkins (1907) also found that the presence of cystine in the diet was beneficial to mice

Cystine in its reduced form cysteine is used by the body as a precursor of taurine



and taurine is converted into the taurocholic acid of the bile and plays an important *role* in the digestion of fats Since the animal body appears to be unable to synthesise cystine it must also be the precursor of glutathione a substance first isolated by Hopkins (1922) and shown by him to be an important agent in many of the oxidations which take place in the living cells of both animals and plants

Histidine and Arginine—Ackroyd and Hopkins (1906) showed that if either arginine or histidine were removed from a mixture of amino acids used as a diet for young growing rats the rate of growth was reduced The absence of histidine was more deleterious than the absence of arginine and ultimately resulted in death When both acids were removed together the disturbance of normal growth was much more marked than when only one alone was missing Their experimental curves are shown in Fig 5 Rose and Cox (1924) do not confirm this work on the necessity for arginine they consider that histidine is the only essential member of this pair of amino acids

Ackroyd and Hopkins discussed the possibility that the function of arginine and histidine might be as precursors of the purines *i e* indirectly of the nucleic acid found in the nucleo proteins Stewart (1925) failed to find any evidence of transformation of either histidine or arginine into purine bodies when perfused in Ringers solution through the surviving liver of the cat Rose and Cook (1925) have however confirmed the function of histidine as a purine precursor Histidine on decarboxylation gives histamine a substance which has a powerful effect on the capillary circulation and may possibly be part of the normal mechanism of the control of the circulation (see Dale and Laidlaw 1910)

Proline —Proline has recently been added to the list of protein units which are necessary in mammalian diet Barnett Sure (1925) records the growth of young rats on two diets adequate as regards all other factors and differing

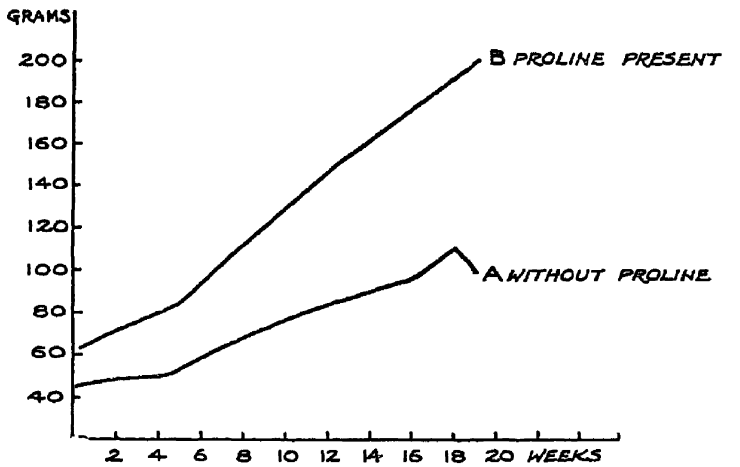


FIG 9 —Influence of proline on the growth of rats on edestin + cystine + lysine In B 0 4 per cent of prol ne added
(From Barnett Sure *Jou rnal of Biological Chemistry* 1924)

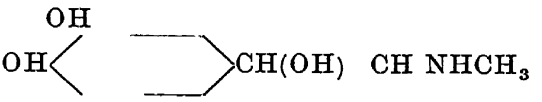
only in their proline content The basal diet contained 6 per cent of edestin which is poor in proline groupings supplemented by 0 4 per cent of cystine and 0 4 per cent

of lysine In the one case (B) 0 4 per cent of proline was given in addition The experimental curves are shown in Fig 9 Further evidence on the biological importance of proline to rats and other animals will have an interesting bearing on the much discussed problem as to whether this imino acid pre exists in native proteins or whether it is formed only during hydrolysis

Tyrosine and Phenylalanine —Abderhalden (1913) found that the adult dog could maintain weight and health on a diet in which the nitrogen was supplied by fully hydrolysed casein to which the tryptophane destroyed in the acid hydrolysis had been restored If tyrosine were removed from the diet however the dog rapidly lost weight and suffered in health Restoration of tyrosine to the diet was followed by recovery of weight and health The evidence of the importance of tyrosine in the diet of other animals is rather conflicting and most of it of an indirect nature Many early experiments on nutrition were made with gelatin which contains no tyrosin It is now recognised that gelatin is also deficient in tryptophane and cystine Nevertheless the evidence of early workers on the effect of tyrosine in supplementing a diet containing gelatin as the sole protein is of considerable value It has been recognised since the end of the eighteenth century that gelatin was an unsatisfactory food material In 1876 Escher showed that if tyrosine were added to gelatin the food value of the latter was increased for pigs Lehmann (1885) did not confirm this for rats and Totani (1916) also found tyrosine not to be essential in the diet of rats being apparently replaceable by phenylalanine This latter unit is present in gelatin to the extent of 1 4 per cent (see p 47 Table I column V) One or other of these two acids however had to be present in the diet Abderhalden (1915) showed that in man gelatin is useless as the sole source of nitrogen but with the addition of tyrosine nitrogen absorption was improved Embden and Balbes (1913) showed that the perfusion of phenylalanine

through the surviving liver of the dog led to the formation of tyrosine and possibly the conflicting nature of the evidence from feeding experiments may be due to the capacity for this synthesis differing from species to species

The function of tyrosine in the body seems possibly to be as the precursor of adrenaline



isolated by Takamine (1901)

Adrenaline is the active principle of the secretion of the suprarenal bodies small ductless glands which lie just above the kidneys Adrenaline has a strong physiological effect on the sympathetic nervous system Bayliss (1918) states that adrenaline is in some way necessary to the activity of the vaso constrictor mechanism *i e* to the contraction of the arterioles and capillaries which supply the various organs of the body with blood Elliot (1912) has shown that certain psychological states such as fright are accompanied by a discharge of adrenaline into the blood This leads to a characteristic pallor (due to the constriction of the capillaries of the skin) an increased blood pressure with increased activity of the heart (Evans and Ogawa 1914) and further to an increased capacity of the skeletal muscles to withstand fatigue (Cannon and Rice 1913) In short the increased bodily efficiency and nervous excitement that characterises a certain degree of fright are secured through the chemical agency of adrenalin

The function of protein in mammalian diet is therefore first and foremost as a source of supply of certain special units or groupings which the body seems unable to synthesise and which it cannot obtain from any other food The function of protein as a source of nitrogen is certainly of secondary importance A protein may therefore be classed as good or poor according to its content in trypto

phane lysine cystine and histidine (with possibly proline and tryosine or phenylalanine) It is obviously also important that these units should not only be present but also available

Digestibility of Proteins

The availability of the amino acid units in a protein depends directly on the capacity of the alimentary enzymes to hydrolyse it

The amino acids are absorbed into the system but peptones and other higher bodies still retaining to some extent the colloidal properties of protein cannot be absorbed and so are lost to the body The more digestible a protein is therefore the greater is its food value The digestibility of a number of proteins is greatly influenced by previous cooking for instance it was mentioned in the previous chapter (p 76) that the raw globulin stizolobin of the Chinese and Georgia velvet beans was only digested to 30 per cent when acted on *in vitro* by the ordinary digestive ferments A lack of feeding power also characterises these two globulins when eaten by stock They were first examined by Links and Johns (1921) because of the unsatisfactory results following their use as a cattle food and it was found later that young growing rats when fed on a standard diet with raw stizolobin as the source of nitrogen lost weight rapidly By cooking the stizolobin the digestibility *in vitro* was raised to 60 per cent and the cooked protein when given in the food of the young rats led to normal growth Many of the vegetable proteins become more digestible after cooking and the same is true of the ovalbumin of egg white Ovalbumin is a protein constitutionally closely allied to lactalbumin yet in the raw state it has practically no food value Mendel and Lewis (1913–14) have shown that in man after a standard meal containing protein in the form of meat there is a rise in the hourly secretion of nitrogen in the urine This rise is due to the amino acids from the hydrolysed protein having been ab

sorbed by the body with subsequent de amination in the liver After a meal containing raw egg white instead of meat the nitrogen excretion only rises slightly Raw egg white is only digested to a very small extent and therefore is evacuated in an almost unchanged condition Cooked egg white however is digested and absorbed by the body and its nitrogen starts to appear almost immediately in the urine Mendel and Lewis s curves of nitrogen excretion after meat raw egg white and cooked egg white are shown in Fig 10

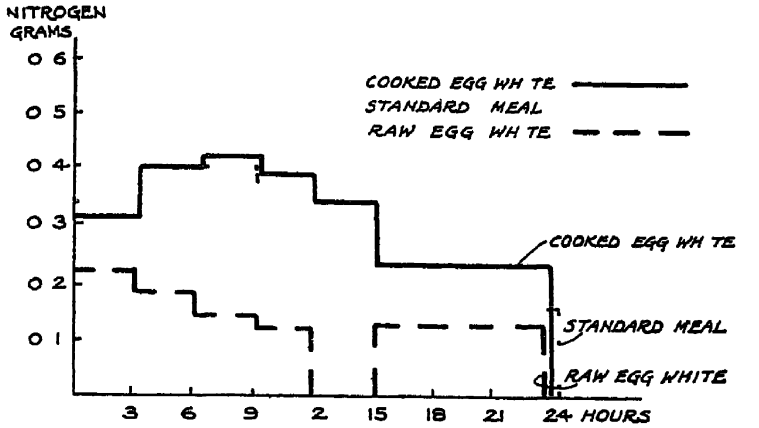


FIG 10 —Excretion of nitrogen after ingestion of cooked and raw egg white compared to that following a standard meal of meat (From Mendel and Lewis *Journal of Biological Chemistry* 1913–14)

Bateman (1916) also showed that raw egg white was not digested by dogs rats or rabbits though egg white coagulated by alcohol or by boiling was readily digested The digestibility of the connective tissue proteins is also increased by boiling and the satisfactory liquefaction of the latter is an important point in dealing with a meat meal Sufficient cooking therefore both of meat and vegetables increases their digestibility over cooking especially over baking of meat tends again towards indigestibility owing to the extreme coagulation of the cell proteins with loss of available surface for enzyme action

Biological Value of the Proteins

It follows that the value of any one protein as a sole nitrogenous constituent in a diet must evidently depend partly on its digestibility and partly on the amino acid units which it contains Rubner (1897) appears to have been the first to realise the possibility of different proteins having different biological values and Thomas (1909) later gave this idea a precise form by defining biological value the number of parts of body nitrogen replaceable by 100 parts of nitrogen in the food stuff The idea of expressing the value of different food proteins on an arithmetical scale is theoretically sound but the practical methods by means of which these values are to be obtained have been the subject of much discussion Martin and Robison (1923) gave a valuable history and criticism of the work recorded on the subject They give as biological values for wheat proteins 31 to 35 and for milk proteins 51 For gelatin the only single protein used by them for experimental work no value could be obtained Their experiments were made on adult males Wagner (1923) working on growing children between the ages of nine and thirteen years gives as values for various proteins—milk protein 80 to 88 egg white 80 maize proteins 54 veal 53 It will be noticed that in no instance was a single protein used in the experiment Wagner makes the valuable suggestion that biological value may vary with the age of the subject he states for instance that only very young children can be brought into nitrogenous equilibrium on milk Wagner has shown that the biological value of one protein may be raised by the addition of another and it is perhaps easily understood that two proteins which in themselves might both be classed as poor may when given together form a good protein diet since the deficiencies of the one may be supplied by the other McCollum Simmons and Pitz (1917) showed that gelatin (in itself a very poor protein) may supplement the food value of

oat protein Osborne and Mendel (1915) showed that gliadin alone can keep young rats at a steady weight but when used as the sole source of nitrogen never leads to growth The addition of gelatin to the diet is followed by immediate growth though gelatin alone cannot even maintain the weight of the rats at a steady level Gelatin as a source of nitrogen has been the subject of much research Cadet de Vaux tried to persuade the poor of Paris during the Revolution that gelatin soup was a satisfactory and nutritious diet the poor however refused to be persuaded and it has now been shown by laboratory experiments that they were thoroughly justified in their attitude Robison (1922) made a prolonged experiment on himself using gelatin only as a source of nitrogen The diet used was made up so as to yield a liberal supply of calories and during the course of experiments his body weight actually increased In spite of this there was persistent loss of nitrogen from the body throughout the whole course of the experiment which ultimately had to be given up for reasons of health

It therefore follows that in calculating a scientific basis for diet the percentage of protein is a value which has very little meaning Every protein has its own feeding value This is well shown in the curves in Fig 11 taken from Osborne and Mendel's paper The curves show the growth of young rats obtained on a diet containing $4\frac{1}{2}$ per cent of protein At this low concentration lactalbumin with its high content of tryptophane cystine and lysine groupings promotes good growth and so does casein when supplemented by cystine but neither casein alone nor any one of the three vegetable globulins is able to promote growth glycinin giving especially poor results It is important to notice that not only is there a necessary minimum value for protein in the diet but also that a certain maximum must not be exceeded With dogs and cats a heavy protein meal is dealt with by vomiting with other animals like rats which do not vomit so easily if at all the ingestion of too

much protein is followed by serious symptoms Osborne and Mendel (1915) give 30 per cent as a dangerous figure Hartwell (1921 1925) has shown that in the lactating rat an excess of protein leads to a diminution of the milk production and the formation of toxic substances in the milk both of which conditions may lead to death of the young

The balance of proteins in the diet especially of the

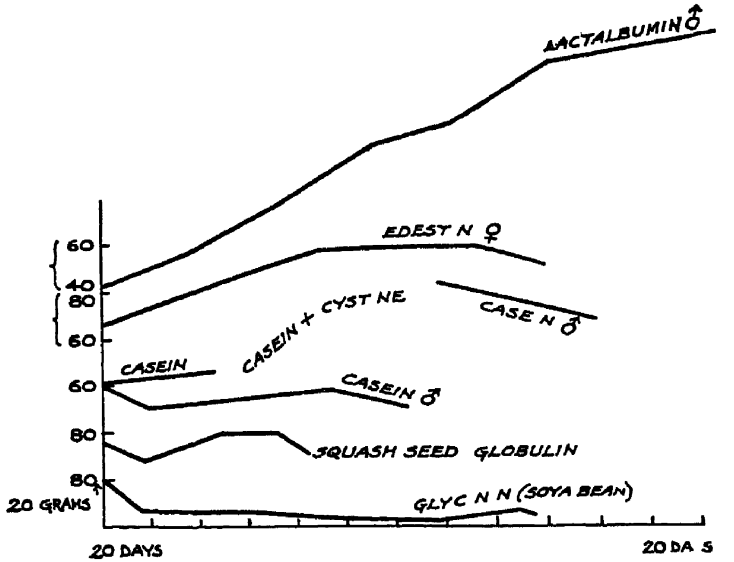


FIG 11 —Growth of rats on diets containing 4½ per cent of protein Lactalbumin gives good growth at this low concentration (From Osborne and Mendel *Journal of Biological Chemistry* 1915)

young growing animal is obviously of the greatest importance both from the point of view of health and of economy Analysis and feeding experiments have shown that the best amino acid balance for the young animal is found in the proteins of milk which is thus proved to be a social necessity Among the cereals the best amino acid balance is found in the proteins of wheat Economic necessity demands that in general the best proteins should be reserved for human consumption It is essential therefore in order to obtain the best results in stock feeding from the proteins available on

the market after the demands of man have been satisfied that a very careful study of these should be undertaken so that any deficiencies in any one type of product could be remedied or balanced by suitable mixing with other material Many difficulties both major and minor in raising young animals satisfactorily will undoubtedly be found due to unsatisfactory diets Hartwell (1925) for instance records that young rats on a diet in which slow growth is being achieved frequently suffer from loss of fur She concludes that the quality or quantity (or both) of the dietary protein is the factor responsible and finds that the cure lies in adding more or better protein There is no doubt that much work still remains to be done on the scientific feeding of stock For instance it was long thought impossible to raise chickens intensively in captivity but Plimmer and Rosedale (1922) have shown that if a diet containing the essential vitamins and a supply of good protein rich in tryptophane is used the chickens grow rapidly and healthily and become mature even sooner than those which have been brought up running on a free range according to the usual method

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CHAPTER VII

THE PROBLEMS OF FOOD PRESERVATION

Autolysis of Tissue Proteins—Bacterial Products from Proteins —The Organic Amines—Methods of Preserving Protein Foods Reduction of Autolysis and Degree of Infection the Principles of Cold Storage the Chemistry of Pickling Method of Dehydration Preservation by Heat Preservation by Smoking Chemical Preservatives

UNDER the conditions of modern civilisation the production of food is generally least intense in those parts of the world where population is densest This leads to the necessity of transporting the bulk of the world s food supply over long distances Moreover while the demands for food on the part of man and his domestic animals are continuous the supply is for the greater part periodic The preservation of protein food is a problem which has required attention since the very earliest stages of civilisation and the solution of which is by no means yet complete The difficulties in preserving protein food of both plant and animal origin arise from two causes firstly the autolytic changes which take place in moribund cells and secondly infection by micro organisms

Autolysis of Tissue Proteins

The autolytic enzymes of the tissues are already present in the living cell the life of which may be regarded as a cycle of chemical reactions These reactions do not cease with the death of the cell on the contrary many of them continue with even greater vigour The essential difference between the living and the dead cell is that in the former all changes are in a reversible cycle whilst in the latter they proceed in one direction only In a dying cell there is a development of free acidity and the hydrogen ion activity

less rapid and less complete In mutton about 20 per cent of the nitrogen is in a soluble form after ten days and in beef less than 15 per cent (Fig 13)

Autolytic action changes the flavour and the consistency of animal tissues and the extent to which it is allowed to proceed varies in practice according to how far these changes are considered desirable or undesirable For instance in game the products of autolytic change are considered to improve the taste of the flesh Game therefore is usually

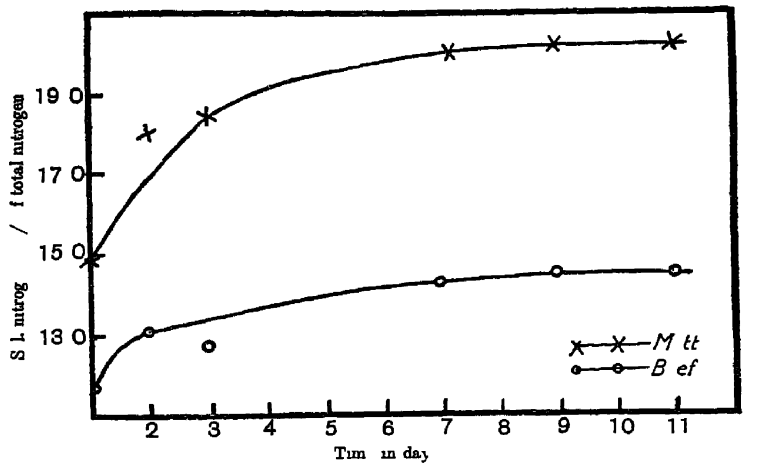


Fig 13 —Aut lysis of beef and mutton at 37
(From Foster and Fearon *Biochem cal Journal* 1922)

kept for a longer or shorter period with the viscera still in the body With ordinary butcher s meat however the strong flavours resulting from autolysis are not usually regarded as desirable and the internal organs are removed before the meat is hung The hanging which is accompanied by a moderate degree of autolysis in the muscles leads to the meat becoming more tender

Bacterial Products from Proteins

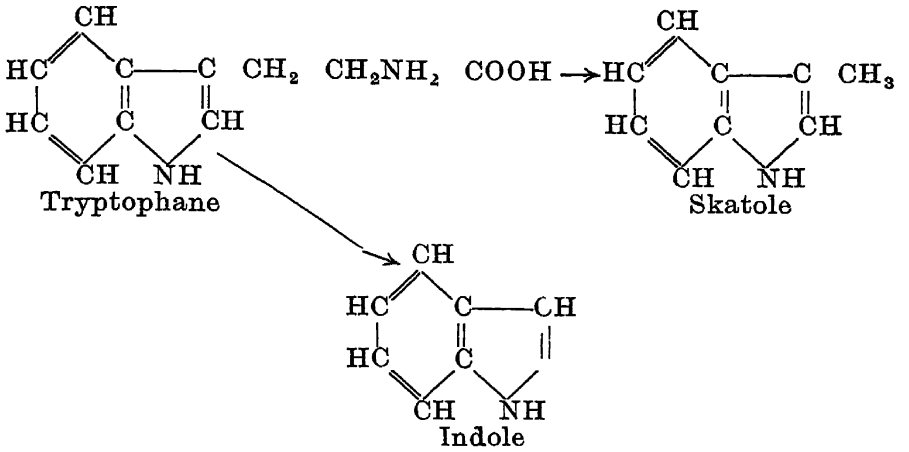
The autolysis that occurs in animal tissues after death has a further influence on the food value due to the fact that

autolysed tissues form a much better medium for the growth of micro organisms than fresh ones Bainbridge (1911) and Sperry and Rettger (1915) have shown in fact that bacteria cannot attack proteins except in the presence of peptone The tryptases of contaminating micro organisms also hydro lyse the cell proteins but other enzymes which are present in them may lead to a further decomposition with in some cases the appearance of toxic end products The types of breakdown of the amino acids by bacteria may follow one of several courses of which the following may be specially mentioned —

(1) *Decarboxylation* with the production of *organic amines* —usually bodies with a strong and offensive smell and sometimes a marked physiological effect

(2) *De amination* with the production of *organic acids*— generally harmless substances but frequently with an un pleasant odour as for instance butyric acid Any con siderable concentration of such acids gives an unpleasant sour taste to food

(3) *Decarboxylation and de amination* with the production of the free *hydrocarbon*—generally a harmless substance but frequently with an unpleasant smell A reaction of this type producing methane from free glycine occurs in the anærobic putrefaction of elastin (Zoja 1897) and fibrin (McCrudden 1910) The best known example of this form of breakdown however is the breakdown of trypto phane which occurs with certain organisms with the pro duction of indole and skatole These are bodies with a strong unpleasant characteristic fæcal smell Skatole has been isolated from putrefying ox brains (Nencl i 1880) indole from putrid meat and many other sources The production of indole from tryptophane is only brought about by certain classes of bacteria and indole production is there fore frequently used for diagnosis for instance *B coli* a normal non pathogenic inhabitant of the intestine produces indole *B typhosus* and *B paratyphosus* both pathogenic



species do not Indole formation therefore is useful in diagnosing typhoid carriers by an examination of the fæces The indole test is also used in examining water for the presence of *B coli* If present there is a possibility that the water may be contaminated by sewage

In addition to the end products of known constitution produced by the ordinary infective bacteria other end products are produced by infections with certain other species For instance *B sporogenes* an organism found in tinned food causes degradation of proteins with the production of large quantities of gas thus causing gas blown tins *B botulinus* is also found in tinned food It has no visible decomposing action on the proteins but produces a highly toxic end substance the constitution of which is unknown

The Organic Amines

A full account of the organic amines will be found in Barger s book The Simpler Natural Bases They are formed by bacteria from amino acids when an alternative source of carbon and nitrogen are both present Kossler and Hanke (1919) consider that on account of their strongly basic nature they are formed as a protective mechanism in media which are becoming progressively more acid They

are usually bodies with a powerful and unpleasant smell and frequently with a strong physiological action

Methylamine CH_3NH_2 derived from glycine the simplest member of the group has been isolated from putrid fish and is the cause of the strong fishy smell (Boel lish 1885 and Morner 1896–97)

Ethylamine $CH_3\ CH_2NH_2$ derived from alanine has been isolated from putrid flour (Sullivan 1858)

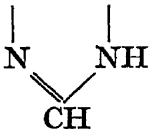
Phenylethylamine $C_6H_5\ CH_2\ CH_2NH_2$ derived from phenylalanine from putrid meat (Bayer and Walpole 1909)

Putrescine $NH_2CH_2\ CH_2\ CH_2\ CH_2NH_2$ is derived from ornithine $NH_2(CH_2)_3CHNH_2\ COOH$ a product of argi nine It has been found in putrid flesh (Brieger 1885)

Cadaverine $NH_2(CH_2)_5NH_2$ is derived from lysine It has been isolated from putrid flesh (Brieger 1885)

Tyramine $C_6H_4OH\ CH_2\ CH_2NH_2$ is derived from tyro sine It has been isolated from putrid meat (Bayer and Walpole 1909) and is a highly toxic substance Tyramine producing bacteria have been isolated from human fæces by Koessler and Hanke (1920)

Histamine $CH{=}C\ CH_2CH_2NH_2$ is derived from histi



dine It has been isolated from the intestinal mucosa (Barger and Dale 1911) and is highly toxic being the well known active principle of ergot produced by the action of an infecting fungus (*Claviceps purpurea*) on the proteins of the rye grain (Barger and Dale 1910) Koessler and Hanke (1924) have shown that the power to convert histidine into histamine is possessed by many bacteria including *B coli communis* and other organisms of the human intestine Dale and Laidlaw (1910–11 1911–12) showed that histamine is a substance which leads to a fall of blood pressure a relaxation of the capillaries and to a

general loss of tone Histamine or histamine like bodies developing either in food through the action of contaminating organisms or in the intestinal contents during the course of digestion probably lead to a large amount of mild chronic poisoning

Methods of Preserving Protein Foods

Since the two factors which lead to the decomposition of protein foodstuffs are autolysis by tissue enzymes and infection by micro organisms there are two fundamental principles that can be employed for the preservation of protein foods The first of these is the inhibition of enzyme or bacterial action by lowering the temperature altering the free acidity or reducing the water content the second is the destruction of enzymes and bacteria accomplished for instance by heating or by smoking In practice the dividing line between one principle and another is not very sharp frequently methods founded on both are employed The technique of the different methods of food preservation is very fully and clearly dealt with by Tressler in his book The Marine Products of Commerce to which the reader is referred for practical details

Reduction of Autolysis and Degree of Infection —With plant and animal tissues which are to be kept for any length of time before being used as food cleanliness and care in handling are of the greatest practical importance Firstly because injury leads to a more acid reaction which favours autolysis and secondly because cleanliness in the work shops is the best way of reducing the chances of bacterial contamination Tressler (1919) gives some interesting figures showing how the first handling of fish affects the length of time during which it can be kept after salting The progress of decomposition was measured by the development of free amino nitrogen This was estimated by a Sørensen titration of an aqueous extract It can be seen how the removal of the easily autolysable viscera helps to preserve the fish

M tl d of Cle g	T m p t e	Amino N f m d d g alt g p d p k lo	C d t f fi h t d of pe od
1 No cleanıng salted hole	79 C	0 77 gm	Badly spoıled bloated
2 Alımentary canal removed		0 63	Spoıled
3 Head cut off all vıscera ex cept mılt or roe removed		0 68	
4 Cleaned perfectly mılt or roe and kıdneys removed blood washed out		0 37	Excellent condıtion

An ınterestıng case where bacterıal contamınatıon can lead to serıous economıc waste occurs ın the condıtıon of rope ın bread Two severe epıdemıcs of thıs occurred durıng the late war and have been reported on ın a paper by the wrıter Clarke and McCrea (1921) Rope ıs a condıtıon ın whıch the bread becomes dıscoloured stıcky and develops a strong and most unpleasant smell Its occurrence durıng the war was due to the use of a quantıty of ınferıor wheat wıth badly fılled graıns that could not be cleaned properly by the scourers of the mıllıng machınes The resultıng flour was therefore hıghly ınfected wıth varıous types of the common saprophytıc organısm *B mesentericus* As a war regulatıon ınsısted that all bread must be kept for twenty four hours before sale thıs heavy ınfectıon resulted ın a number of ınstances durıng the summer months of the bread putrefyıng under the actıon of the bacterıa Thıs putrefac tıon was accompanıed ın all cases by a breakdown of the wheat protein and ın a few cases only by decompositıon of the starch The bread although quıte harmless became useless as food owıng to the unpleasant physıcal charac terıstıcs whıch ıt developed

The Prıncıples of Cold Storage—Thıs method ıs un doubtedly the most ımportant of all ways of preservıng food sınce ıt results ın the fewest changes ın the food ıtself and preserves the vıtamıns When the technique of

cold storage has been fully worked out cold stored food should not differ materially from fresh food The principle of cold storage is that by lowering the temperature the rate of autolysis and growth of contaminating organisms is reduced Foster and Fearon (1922) give curves showing the rate of autolysis of beef and mutton at 37° 6° and 0° the two former are reproduced in Figs 13 and 14 (pp 101 and 108) At the last temperature a slow autolysis is still in progress though greatly reduced in rate This slow autolysis has been supposed to account for the loss of flavour which takes place in cold stored meat and has been associated with the disappearance of the carnosine of the muscle Carnosine is a dipeptide β alanyl histidine It is tasteless itself but is supposed to be associated with flavour Clifford (1922) gives figures showing the carnosine content in fresh (English) and cold stored imported meat

	Ca t t pe e t	
	E gl h me t	Imp t d m t
Beef	1	0 3ɔ
Veal	1	0 3ɔ
Mutton	0 37	0 15
Lamb	0 4	0 1ɔ

In using the method a technique has to be evolved which is suitable for cooling rapidly material with a cellular structure If the tissues are cooled too slowly large crystals of ce are formed in and between the cells and on subsequent thawing the tissue drips badly—that is the cell contents escape through the damaged walls By very rapid freezing the ice forms as innumerable tiny crystals which do not damage the cell structure Rapid freezing and slow thawing are essential for the successful handling of food preserved by cold The drip which escapes from cold stored tissues on thawing is a source of loss of nitrogen Callow (1925) has shown that

in the drip from thawed cod there is actually coagulable protein The damage to the tissues in badly frozen samples has a very marked influence on the course of autolysis which takes place after the meat leaves the cold chambers In Fig 15 are shown the curves of autolysis obtained by Foster and Fearon for beef which has been kept in cold storage under various conditions The usual method of preserving beef has previously been storage in cold air chambers at about — 8 C It can be seen that such material (Curve B

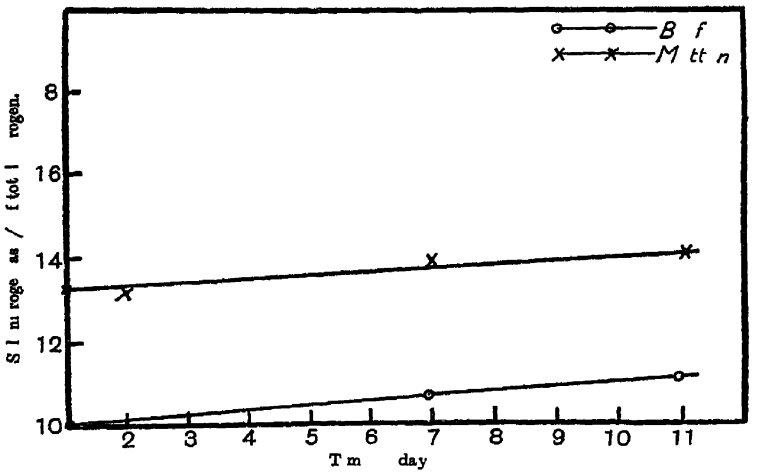


FIG 14 —Autolysis of beef and mutton at 6
(From Foster and Fearon *Biochemical Journal* 1922)

Fig 15) after removal from the cold chamber undergoes autolysis at a rate considerably greater than that of unfrozen meat If the beef is first rapidly cooled in air at — 18 C and afterwards is placed in the cold air chambers at — 8 C autolysis though still rapid is reduced in rate (Curve D) If the beef is rapidly frozen in brine at — 8 C and is kept in the brine vessels the course of autolysis after thawing is not very different from that of fresh beef (Curve E) The work of Foster and Fearon on the autolysis of frozen beef has been extended by Callow (1925) to the muscles of the cod Cod muscle slowly frozen in air at — 11 C auto

lysed more rapidly after freezing than an unfrozen control whilst cod muscle rapidly frozen in brine at — 19 C showed no difference from the control

Autolysis encourages putrefaction by giving bacteria a good medium for growth and defective methods of cold storage lead as is well recognised to a more rapid putrefaction in the thawed tissues Frozen meat game fish etc

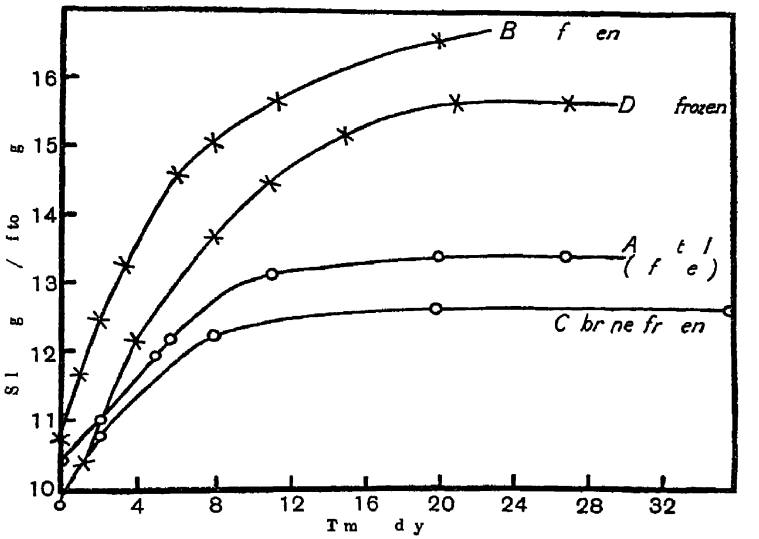


FIG 15 —Autolys s at 37 of fresh beef and of beef previously frozen slowly in air (A and D) o qu ckly in b ne (C)
(From Foster and Fearon *Biochemical Journal* 1922)

keep badly and are therefore never removed from the chambers until wanted

The Chemistry of Pickling —The optimum reaction for the autolytic enzymes of muscle is given by Chen and Bradley (1924) as *p*H 4 5 — 5 0 The limiting reaction for the growth of nearly all bacteria is *p*H 5 0 — 5 5 and for most moulds is about 3 5 — 3 0 By raising the acidity therefore it is possible to inhibit autolysis and the growth of micro organisms This is the principle of pickling The reaction of a pickling liquid may be as high as *p*H 2 8 It will be seen

later that all tissues swell in acid solutions and to prevent this salt is always added to a pickling liquor Acetic acid is always used for pickling and to be effective must be present to the extent of 15 per cent It is usually added as vinegar Pickling was one of the early methods of preserving food for long journeys especially in the days of the sailing ship It has the advantage of requiring no accessory apparatus It can be used to preserve meat fish and vegetables There is still a large trade in pickled herrings between the Scottish ports and Central Europe Meat and vegetables are not usually pickled nowadays for the sake of storage and trans port but a number of methods of pickling in which spices are added are in common use for preparing special table delicacies such as the Bismarck herring soused mackerel pickled cabbage etc etc Pickles of this type are cooked before pickling The pickled pork and beef of a former era were pickled before cooking

Method of Dehydration —The preservation of animal and vegetable tissues by dehydration is the simplest and the oldest method known It is used by the most primitive people for storing in a time of plenty against a time of scarcity or for journeying under difficult conditions It is obvious that dehydration by direct evaporation under conditions of sun light and warmth is only possible in countries with a hot dry climate hence it is in common use among people of poor culture in tropical regions Under more civilised conditions meat and fish are never prepared for storage by mere dehy dration by heat although this method is employed in the manufacture of dried milk Milk is a fluid containing casein and large quantities of lactalbumin It contains in addition fat in the form of finely divided globules and various crystalloid constituents such as sugar and salts Milk is an emulsion and its physical condition is largely maintained through the action of the lactalbumin which seems to act as a protective colloid Now lactalbumin is a protein which is very readily coagulated by heat that is turned into an in

soluble form After heat coagulation the lactalbumin can no longer assist in maintaining the emulsion and the problem of the manufacture of dried milk is how to get rid of the large quantity of water present in the milk without simultaneously coagulating the lactalbumin and breaking up the emulsion so that it cannot be restored The method employed nowa days is to run the milk in the form of a very thin layer over hot rollers or to let it fall as a fine spray down a hot tower By this method the surface exposed is enormously increased and evaporation becomes very rapid Since the coagulation of proteins by heat is a time reaction if the evaporation can be made to occur sufficiently quickly then the water will have been driven off before coagulation has proceeded far enough to be deleterious

Preservation by evaporation to dryness is also the method frequently used for preparing dried eggs The only advan tages to be obtained by drying such products as milk and eggs are cheapness and convenience of transport There is no doubt however that for their dietetic value the natural products are still in spite of enormously improved methods preferable to the dried ones

In hot countries many fruits are preserved by drying Currants and grapes for instance are treated in this way Seeds such as peas and lentils are also dried and the method is used for some vegetables Drying leads to a reduction of the vitamin content of vegetables especially of the anti scorbutic vitamin

In temperate climates and in slightly more advanced con ditions of civilisation dehydration as a method of preserva tion for meat and fish is usually brought about by salting Either dry salting or brining *i e* steeping in saturated crude solutions of sodium chloride may be used In either case the water from the tissues dialyses outwards and some of the sodium chloride diffuses inwards Both these streams of diffusion lead to the reduction of the proportion of water in the cells and in the resulting solution the cell enzymes

become greatly reduced in activity A 4 per cent concentration of salt in the tissues noticeably retards autolysis and bacterial growth A 20 per cent concentration inhibits both to such an extent that the food can be preserved for long periods at ordinary temperatures Salting is sometimes followed by smoking as a method of cure

Preservation by Heat—In using heat as a preservative the tissue enzymes and contaminating micro organisms are (or should be) destroyed during the process The ordinary processes of cooking are sufficient to keep most animal and vegetable food for a few days except under the most unfavourable circumstances Where preservation for any length of time is desired the action of heat is supplemented by sealing the sterilised food under anærobic conditions into jars or tins This prevents re contamination and the development of ærobic bacteria but will not check the growth of any anærobic bacteria which may have survived The problem of preserving food by heat lies in choosing a temperature and time of heating sufficient to destroy bacteria without causing too much structural alteration in the food material Nearly all foods whether of animal or vegetable origin can be preserved by canning The method is used for meat (beef ox tongues etc) fish which may have been cooled by steam (tinned salmon etc) or sometimes in oil (sardines) fruit and vegetables It is sometimes used after a certain amount of evaporation for milk The great advantage of tinned food is that it can be kept indefinitely under the most primitive social conditions Preservation by heat in nearly all cases destroys the vitamin content of the food so handled

Preservation by Smoking—Preservation of food by drying it by evaporation in the sunlight is the most obvious and the most primitive method It is not a very much further step to preserving it by drying in the smoke of a wood fire When fish or meat is cured by smoking partial dehydration takes place but in addition the surface of the tissue undergoing treatment becomes impregnated with phenolic sub

stances present in the smoke These substances inhibit the growth of micro organisms and smoked food therefore is preserved both by the drying action and by chemical treatment of the surface Bacon Scotch kippers and Finnan haddocks are examples of foods where smoking is a part of the method of cure Smoked foods have only a limited resistance to contaminating bacteria or moulds Vegetables are never preserved by this method

Chemical Preservatives —It is in certain trades a common practice to add antiseptics to food preparations for the purpose of preventing the growth of micro organisms The persistence with which moulds will grow on the surfaces of tissues even at very low temperatures led at one time to the spraying of meat in cold storage chambers with a weak solution of formaldehyde Bacon is also sometimes treated on the surface with boric acid The question as to how far it is permissible to preserve food by means of antiseptics cannot be dealt with in this book and reference should be made to the Report of the Parliamentary Committee on Preservatives and Colouring Matter in Food (1925)

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PART II

CHAPTER VIII

THE COMBINATION OF PROTEINS WITH ACIDS AND ALKALIS

Solubility of Proteins in Acids and Alkalis—Minimum Solubility and the Iso electric Point—Salt Formation and Protein Constitution—Combination with the +ve and −ve Ions of Acids—Acid Binding Power and the Free Amino Groups of Proteins—Alkali Binding Power and the Free Carboxylic Groups—Ionisation Constants—Concentrated Solutions of Acids and Alkalis—The Reactions of Proteins and Acids near the Iso electric Point—The Hermaphrodite Ions of Proteins—Strong and Weak Acids and Bases—Combination of Proteins with Acids and Alkalis in the presence of Salts—Determinations of the Iso Electric Points of Proteins by Titration Curves—Reactions with Salts in the presence of Acid and Alkali—Protein Buffers

Solubility of the Proteins in Acids and Alkalis

THE chemical constitution of the protein molecule dealt with at length in Part I influences very definitely the general properties of the proteins The most active groups in the molecule are the basic amino and the acid carboxylic groups and it is therefore not surprising to find that hydrogen and hydroxyl ions have a predominating influence both on the solubility of proteins in water and on the physico chemical properties of the resulting solutions

The mutual solvent action between acids and all alis and proteins has been known for very many years Heintz (1853) states that more lime is dissolved by solutions of gelatin than by water Kuhne (1868) found that more gelatin Osborne (1902) that more edestin is dissolved in the cold by dilute acid and alkali than by water alone and Hardy (1899 1905) that coagulated egg white or globulin both of which are insoluble in water readily dissolve in dilute acids and alkalis Fischer and Coffman (1918) state that even

in the presence of buffer salts the solubility of gelatin increases with the concentration of acid or alkali The quantitative relationship between the concentration of acid or alkali in a system and the amount of protein dissolved has recently again been demonstrated by Fairbrother and Swann (1922) in a series of experiments in which gelatin was left for ten days in contact with dilute hydrochloric acid or sodium hydroxide solutions Equilibrium having been attained analysis of the solutions gave the results shown on p 117 Table IV

These experiments demonstrate three important points firstly that with increasing concentration of acid or alkali more and more gelatin goes into solution secondly that acid or alkali disappears from the solution and thirdly that there is a definite point of minimum solubility in very dilute acid That the gelatin is dissolved and not hydro lysed under these conditions is proved by the fact that it can be recovered unchanged (Jordan Lloyd 1920 Fair brother 1924) The behaviour of gelatin is typical of most proteins though the amount of acid or base needed for complete solution differs in every case With commercial casein the gliadins and the glutenins there is a wide zone of acid concentration over which the protein does not go into solution although it combines with the acid present in an amount depending on the concentration of the latter (L L van Slyke and Hart 1905 van Slyke and van Slyke 1907 Osborne 1902) The existence of a well marked point of minimum solubility over a narrow range of hydrogen ion concentration is however characteristic of most proteins

Minimum Solubility and the Iso electric Point

Hardy (1899 1905) pointed out that proteins go into solution in acids as positively charged colloids in alkalis as negatively charged ones At the point where they carry no electric potential towards the surrounding solvent called by

TABLE IV

Hydrochl ric acid 1 gm of gelat n n 100 c c

Init al concentrat on of acid as normality	0 500	0 200	0 100	0 050	0 020	0 010	0 0067	0 0050	0 0020	0 0010	0 0002	0 0000
Final concentrat on of ac d	—	0 1986	0 0970	0 0450	0 0134	0 0038	0 0019	0 0009	0 0006	—	—	0 0000
Gelatin dissolved Grams	1	0 24	0 14	0 10	0 08	0 08	0 05	0 03	0 02	0 01	0 03	50 0

S d um Hyd xide 1 gm f g lat n n 100 c

Init al concentration of alkal as normality	0 150	0 025	0 020	0 0125	0 0100	0 0062	0 0050	0 0020	0 0010	0 0000	—	—
Final concent at on of alkal	—	0 0188	0 013	0 0068	0 0049	0 0024	0 0017	—	—	0 0000	—	—
Gelat n dissolv d Grams	1	0 22	0 18	0 11	0 08	0 07	0 06	0 05	0 05	0 05	—	—

him the iso electric point solubility is at a minimum The value of the hydrogen ion concentration at the iso electric point is now known to be a physical constant for each individual protein and moreover Cohn (1922) has shown that if a pure protein (*i e* one free from electrolytes) be placed in contact with distilled water (free from carbon dioxide) the amount of protein dissolved and the reaction (*p*H) produced by its ionisation are also physical constants The iso electric points of a number of proteins are given in Table V column II p 130

Salt Formation and Protein Constitution

The theory that the increased solubility of proteins in acid or alkaline solutions is due to the formation of protein salts appears to have first been put forward by Nasse (1889) and later by Bugarsky and Liebermann (1898) who showed that in an acid solution acid was actually combined with the protein and stated that the protein salts produced were ionised in solution in the same way as the salts of crystalloids Later Hardy (1905) suggested that the mechanism of the salt formation was localised in the free amino and carboxylic groups of the protein according to the following schemes in which HS is taken as a general expression for an acid BOH for an alkali and $R\begin{smallmatrix}NH_2\\COOH\end{smallmatrix}$ for a protein —

(*a*) In acid solution

$$R\begin{smallmatrix}NH_2\\COOH\end{smallmatrix} + HS \rightleftarrows R\begin{smallmatrix}NH_2\ HS\\COOH\end{smallmatrix} \rightleftarrows \left(R\begin{smallmatrix}NH\ \ H\\COOH\end{smallmatrix}\right)^{+} + S^{-}$$

(*b*) In alkaline solution

$$R\begin{smallmatrix}NH_2\\COOH\end{smallmatrix} + BOH \rightleftarrows R\begin{smallmatrix}NH_2\\COOB\end{smallmatrix} + HOH$$

$$B^{+} + \left(R\begin{smallmatrix}NH_2\\COO\end{smallmatrix}\right)^{-}$$

Final proof that salt formation is particularly connected with these two groups has had to wait for the development of a fuller knowledge of the chemistry of protein constitution than was available at the time it was enunciated Evidence of the extent of combination of the positive and negative ions of an acid with dissolved protein was however soon forthcoming

Combination with the +ve and −ve Ions of Acids

The mechanism of salt formation between proteins and acids suggested by Hardy corresponds to the theory of the formation of ammonium salts put forward by Werner (1913) *i e* the trivalent nitrogen atom binds the acid molecule by means of two extra valencies one +ve the other −ve to form salts in which the −ve ion of the added acid is again ionisable

It is obvious therefore that if Hardy s theory of salt formation and ionisation is correct then the addition of protein to an acid HS or $H^+ + S^-$ should lead to a considerable reduction in the concentration of the H^+ ion and to very little in that of the S^- ion

The method generally adopted for measuring ionic con centrations is that of determining the electric potential developed between a standard solution containing a known concentration of the ion under investigation and the experi mental solution containing the unknown concentration Thus the electrical potential developed between a surface of platinum black saturated with hydrogen (potentially solid hydrogen) and any solution containing hydrogen ions is a measure of the concentration of the hydrogen ions The development of the necessary technique for measuring hydrogen ion concentration is due largely to the work of Sørensen (1909) and for a full account of both theory and practice the reader is referred to his original papers to Michaelis Wasserstoffionen konzentration (1914) or

to Clark The Determination of Hydrogen Ions (1923) Short accounts are given in Findlay s Physical Chemistry for Medical Students and Michaelis Practical Physical and Colloid Chemistry (1925) Although these deal mainly with the measurement of the concentration of the hydrogen ion it must be remembered that the principle of the con centration chain can be applied to any ion provided a suitable electrode can be found

Manabe and Matula (1913) made use of this method for examining the relations of the positive and negative ions of hydrochloric acid to the proteins horse serum egg albumin and gelatin By measuring the actual concentra tions of the hydrogen and chlorine ions in the presence of constant protein and varying total acid concentrations they obtained a measure of the amount of each ion removed from the solution by the protein They measured the concentration of the hydrogen ion by means of a platinum hydrogen electrode that of the chlorine ion by a mercury calomel electrode The concentration of the hydrogen ion determines that of the free acid and the difference between total and free acid gives a measure of the acid bound by the protein Thus $n = n - \frac{C_H}{\alpha}$ where α is the degree of ionisation of a strong acid at a concentration of C_H C_H is the concentration of free hydrogen ions measured by the hydrogen electrode n is the total acid concentration and n therefore the amount of combined acid This formula is only used for strong acids at a concentration of $< 0\ 05$ N The chlorine bound was determined by a similar calculation and is a measure of the concentration of the unionised gelatin salt The difference between free hydrogen ion and free chlorine ion is of course a measure of the concentration of the ionised gelatin salt

Manabe and Matula found that with increasing concentra tion of acid increasing amounts of hydrogen were bound by the protein though the ratio of hydrogen ion bound to total

acid concentration became less and less Further up to an acid concentration in the system of 0 03 Normal the amount of chlorine bound was very considerably less than that of the hydrogen bound In the presence of more acid the amounts of the two ions bound tended to become more equal

Hitchcock (1922/3) measured the combination of the

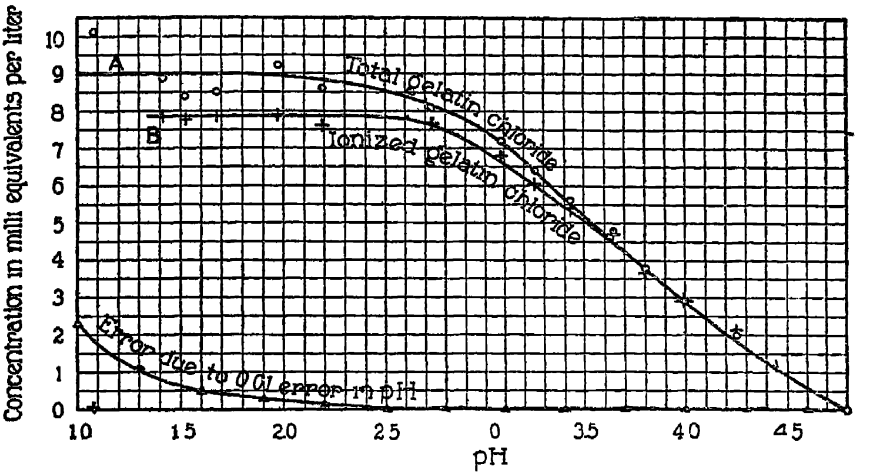


FIG 16 —The influence of hydro en on concentrat on on the combinat on of gelat n and hydrochloric acid Curve A shows the ncrease in the total acid concentration necessary to maintain the same *p*H in an acid solution after the add tion of 1 p r cent of gelatin Curve B shows the excess of chlorine ions over hydro en ions in the s lut on Tempera ture = 25

(From Hitchcock *Journal of General Phys ology* 1922/3)

hydrogen in a number of protein hydrochloric acid systems by means of the hydrogen electrode and the combination of chlorine in the same systems by means of a silver silver chloride electrode He expressed his results by a different method from Manabe and Matula His curve of hydrogen ion bound is shown in Fig 16 under the title Total gelatin chloride and his curve showing the excess of free chlorine ions over the free hydrogen ions as Ionised gelatin chloride

It can be seen from Figs 16 and 17 for gelatin hydrochloric acid and globulin hydrochloric acid systems that in dilute concentrations of acid (pH>2) the protein salts exist in an almost completely ionised condition The work of Manabe and Matula and of Hitchcock justifies the belief that proteins form salts with acids which ionise in the same way as ordinary salts Their experimental results have been

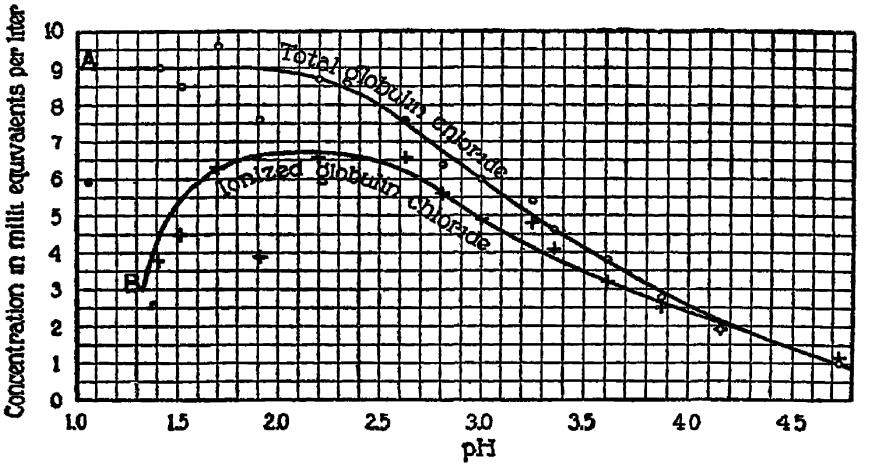


FIG 17 —The influence of hydrogen on concentration on the combination of globulin and hydrochloric acid Curve A shows the increase in the total acid concentration necessary to maintain the same pH in an acid solution after the addition of 1 per cent of globulin Curve B shows the excess of chlorine ions over hydrogen ons in the solution Temperature = 25

(From Hitchcock *Journal of General Physiology* 1922/3)

described in some detail because Robertson has suggested that protein salts ionise not into one colloidal and one diffusible ion but into two colloidal ions This hypothesis which has been the starting point of a large amount of experimental work is developed in detail in Robertson s book The Physical Chemistry of the Proteins (1918)

Further work on the fixation of the hydrogen ion by proteins has been published by Wintgen and Kruger (1921) and Wintgen and Vogel (1922)

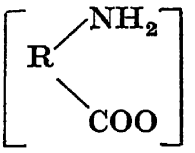
Acid Binding Power and the Free Amino Groups of Proteins

The free amino groups of a protein may be considered to come partly from the lysine and partly from the arginine groups which it contains (see Chapter IV) The former can be removed by the action of nitrous acid (Van Slyke and Birchard 1913) but the latter are not readily affected (Van Slyke 1911) The acid binding power of a protein if due to the free amino groups should therefore be affected by nitrous acid in accordance with the proportion of these two units which it contains In the case of gelatin this proportion is four lysine to five arginine groupings in a molecule with a weight of 10 300 (Jordan Lloyd 1920) Bracewell (1919) calculated that if the combination of hydro chloric acid with gelatin occurs at the free amino groups of lysine and arginine and in equal amounts at each group 1 gram of gelatin should combine with 0 00085 equivalents of acid He found by a titrimetric method that 0 00070 equiva lents were removed by powdered gelatin from a supernatant solution of hydrochloric acid Jordan Lloyd and Mayes (1922) by an electrometric titration found 0 0010 equiva lents Hitchcock (1923/4) 0 00089 for gelatin and 0 00044 for de aminated gelatin moreover the loss of acid binding power in the latter calculated as loss of amino groups is accurately accounted for by the nitrogen produced during de amination by nitrous acid Blasel and Matula (1914) had already shown that at the same total concentration of acid in a system the fixation of the hydrogen ion by de aminated gelatin is about half that of untreated gelatin but that the fixation of the chlorine ion is practically the same in both systems The quantitative relations between the normal and the de aminated protein therefore confirm the theory that the formation of ionisable salts of the ammonium type takes place at the free amino groups of the lysine and arginine units of the protein molecule The possibility that the

groupings of histidine and hydroxylysine when present can also function as acid binding groups will ultimately have to be considered

Alkali Binding Power and the Free Carboxylic Groups

The combination of proteins with bases has been the subject of a considerable amount of experimental work but very few data are available which give the variation of base bound to hydroxyl ion concentration over a continuous wide range Atkin and Douglas curve for the combination of gelatin and sodium hydroxide from *p*H 4 7 to 13 is shown in Fig 18 (p 132) This shows that under these conditions com bination of gelatin and base takes place in two stages the first in which 1 gm combines with about 30×10^{-5} equivalents of base the second in which 1 gm combines with 80×10^{-5} equivalents This discontinuous type of combination between proteins and bases is indicated again from other experiments casein can combine with either calcium or magnesium hydroxide to the extent of $11\ 25 \times 10^{-5}$ equiva lents of base to 1 gm remaining the while completely insoluble it can then combine with further base to produce soluble derivatives $22\ 5 \times 10^{-5}$ equivalents being necessary just to carry the casein into solution (van Slyke and Bosworth 1913 van Slyke and Winter 1914) and 180×10^{-5} equiva lents being the maximum combining power (Robertson 1910) The *p*H value which marks the transition point between the formation of the insoluble and the soluble calcium derivatives is unknown but must be well on the acid side of absolute neutrality since at neutral to litmus 1 gm of casein binds 50×10^{-5} equivalents of base (Robert son 1910) The complete insolubility of the first calcium casein compounds formed and the ready solubility of the later ones raises the doubt as to whether the two stages of combination are chemically analogous Do proteins only form compounds with bases of the ionisable salt type as



as suggested by Hardy or do they also form non ionising compounds of another type possibly by substitution in a hydroxl group ?

The basic binding power of casein and zein has been critically examined by Cohn and Berggren (1924/5) and Cohn Berggren and Hendry (1924/5)

Evidence has been given in Chapter IV that the terminal carboxylic groupings of the protein molecule are derived entirely from the di carboxylic acids present in the molecule These are aspartic glutaminic and β hydroxyglutaminic acids In the vegetable proteins the terminal carboxylic groupings exist mainly in the form of acid amides in the animal proteins mainly in the free state In either case if protein salt formation with bases occur only at the free carboxylic groups then the basic power of a protein should be chemically equivalent to its molecular content in di carboxylic acids *minus* its content in ammonia Cohn and Berggren (1924/5) calculate that if the molecular weight of casein be taken as 12 800 (minimum weight equivalent to a content of one molecule of tryptophane) then the molecule contains 19 molecules of glutaminic acid 8 of β hydroxyglutaminic and 4 of aspartic making a total of 31 terminal carboxylic groups Since casein yields 12 molecules of ammonia which are probably present as acid amides this brings the number of free carboxylic groups available for salt formation to 19 Cohn and Berggren find that casein prepared without contact with strong alkali combines with sodium hydroxide in the proportion of 0 0014 equivalents of base to 1 gram *i e* it behaves as if it has 18 acid valencies If allowed to come in contact with strong alkali during preparation 1 gram of casein combines with 0 0018 equivalents of base *i e* the protein behaves as if it has 24 acid valencies They suggest that these extra valencies may come from the

phosphorus in the molecule Cohn Berggren and Hendry (1924/5) similarly calculate that there should be six free carboxylic groups in zein and that 1 gram of protein should combine with 0 0030 equivalents of sodium hydroxide Actually they obtained 0 0028 equivalents per gram with one preparation 0 0031 with another In a molecule of gelatin weighing 10 300 aspartic acid forming 3 4 per cent contributes 3 carboxylic groups glutaminic acid forming 5 8 per cent 4 making a total of 7 But the gelatin molecule yields three molecules of ammonia (Jordan Lloyd 1920) hence the basic binding power should be 0 004 equivalents for 10 gm Atkin and Douglas (1925) find 0 0030 for a first stage of titration 0 007 for a second stage The evidence therefore suggests that while proteins mainly form salts with bases by combination at the free carboxylic groups other types of combination may also occur The question becomes of great physiological importance in determining the condition of calcium in the blood Cameron and Moor house (1925) give a review of the literature of this subject As a result of their own experiments in which the ionic calcium in blood was estimated by precipitation as calcium oxalate they concluded that about half the calcium present is in the form of a non diffusible non ionisable compound though they reject the view that this may be a calcium protein compound Neuhausen and Marshall (1922) however who determined the concentration of calcium and sodium ions by means of the calcium and sodium electrode respectively found 80 per cent of the calcium present in an un ionised form though the whole of the sodium was ionic They consider that the un ionised calcium is present as a compound with the plasma proteins It will be seen in the later chapters of this book that those physical properties of the proteins such as swelling viscosity etc which can be attributed to the formation of soluble ionisable salts are not symmetrical about the iso electric point but have for the most part a latent period between this point and absolute

neutrality which is only partly to be ascribed to the use of logarithmic scale of notation for the hydrogen ion concentration

Ionisation Constants

The electrometric titration curves of the proteins are similar in type to those of amino acids dipeptides urea and other weak bases (see Harris 1924) From such curves it is possible to calculate the equivalent combining weight of the protein in acid and in alkaline solution and its ionisation constants as a base and as an acid respectively It must be borne in mind however that although for amino acids such constants apply to individual groups and therefore have a precise meaning for large multivalent molecules like proteins they can only be regarded as average values for a number of groups

It is not proposed to give here a full account of the theory of titration for which the reader is referred to the works of Sørensen Michaelis or Clark already quoted but attention may be called to a simple generalisation derived from the law of mass action which is useful for obtaining the approximate value of the ionic dissociation constants of proteins

The ionisation of a weak acid in watery solution may be expressed as

$$[H] \times [S] = K\,[HS] \qquad (1)$$

where [HS] is the concentration of the unionised acid [H] and [S] of the two ions into which it dissociates

(1) may be re written

$$[H] = K\,\frac{[HS]}{[S]} \qquad (2)$$

But in the titration of a weak acid by a strong base the concentration [HS] of the unionised acid is very nearly equal to the total acid concentration and the concentration [S] of the negative ion is very nearly equal to the concentration

of the salt formed during titration since this will be highly ionised Hence (2) may be written

$$[H] = K\left(\frac{\text{acid}}{\text{salt}}\right) \tag{3}$$

Now when half the acid has been neutralised in titration the concentration of the acid left is equal to that of the salt formed *i e*

$$[HS] = [S] \quad \text{and} \quad \frac{[HS]}{[S]} = 1$$

Hence at half titration

$$[H] = K$$

or *at half titration of a weak acid the hydrogen ion concentration is numerically equivalent to the ionisation constant* Now the hydrogen ion concentration is usually expressed as pH the negative logarithm of [H] *i e* $pH = \dfrac{1}{\log [H]}$

If $[H] = K$

Then

$$\log [H] = \log K_a$$

$$pH = \frac{1}{\log K}$$

$$\text{or } \log K_a = \frac{1}{pH} \tag{4}$$

Similarly it can be shown for a weak base that at half titration

$$K_b = [OH]$$

$$\text{or } \log K_b = \frac{1}{p_{OH}}$$

But p_{OH} is more conveniently written in the form

$$p_{OH} = pK_w - p_H$$

where pK is the negative logarithm of K_w the ionisation constant of water

Hence $$\log K_b = \frac{1}{pK_w - p_H} \qquad (5)$$

or *at the half titration of a weak base the hydroxyl ion concentration is numerically equal to the ionisation constant of the base* or the reciprocal of the logarithm of the ionisation constant is equal to the ionisation constant of water minus the hydrogen ion concentration

It follows therefore that from the titration curves of the proteins it is possible to read off the pH at half titration and so to obtain values for K and K_b by means of equations (4) and (5) The use of the method involves the assumption that at half titration protein salts are fully ionised An examination of Figs 15 and 16 shows that gelatin chloride is completely and globulin chloride about 85 per cent ionised under these conditions The method is therefore justifiable in titrations with acids With bases experimental justification is lacking and moreover with most proteins an accurate titration curve with these is not so easily obtained as with acids If however K_b and I the pH value of the iso electric point of the protein are known K can be deduced from the equation —

$$I = \sqrt{\frac{K}{K_b}}\ K_w \qquad (6)$$

Titration of a protein with an acid may therefore be used to evaluate both K and K_b

In Table V are given the ionisation constants for several proteins mainly calculated from Hitchcock s experimental data by means of equations (5) and (6) The equivalent weights of the proteins in acid and alkaline solution are included in the table and corresponding data obtained by the same method for a few amino acids For comparison the data for a few acids and bases have been added The protein data have mainly been derived from titration curves with hydrochloric acid In the case of calculations from Hitch cock s curves which were made at 25 $pK_w = 13\ 89$

Physical Constants

Protein	Isoelectric point *p*H	Equivalent weight Acid
Crystallised egg albumin	4·8 ( )	1150 ( )
Electrically flocculated egg albumin	4·8 ( )	—
Serum albumin	4·7 ([5])	590 ( )
Denatured serum albumin	5·4 ( )	—
Myogen (guinea pig)	6·3 ( )	—
Denatured myogen (guinea pig)	6·3 ( )	—
Myogen (frog)	6·0 ( )	—
Serum globulin	5·52 ([8])	1100 ( ) 1400 ([9]) 1700 ( )
Edestin	5·0 to 6·0 ( )	820 ([13])
Oxyhæmoglobin	6·7 ([1])	—
Myosin (guinea pig)	5·1 ( )	—
Fibrin	about 6·8 ([16])	—
Gelatin	4·7 ([7])	1180 ( ) 1100 ( )
	4·7 to 5·5 ( )	1000 ( )
Electrically flocculated gelatin	4·9 ( )	—
Deaminated gelatin	4·0 ( )	2270 ([2])
Collagen	4·8 ( )	—
Collagen (ash free)	5·5 ( )	—
Casein	4·6 ( )	1510 ( )
Glycine	6·6 ( )	75
	6·1 ([0])	
Arginine	10·5 ([1])	93
Aspartic acid	3·0 ( )	133
Ammonium hydroxide	—	—
Acetic acid	—	—
Carbonic acid	—	—

([1]) Sørensen 1915
( ) Hitchcock 1922/3
( ) Cohn Hendry and Prentiss 1925
([4]) Pauli and Modern 1925
([5]) Michaelis and Davidsohn 1911
([6]) Pauli and Hirschfeld 1914
( ) Weber 1925
([8]) Michaelis and Rona 1910
([9]) Hitchcock 1922/3 1
([0]) Adolph 1924
( ) Adolf 1923
([2]) Robertson 1907
([13]) Hitchcock 1924
( ) Michaelis and Davidsohn 1912
([1]) Michaelis and Airila 1921
([1]) Fischer and Hooker 1918
( ) Michaelis and Grineff 1912
([8]) Gerngross and Bach 1924
([9]) Atkin and Douglas 1924

at Maximum Combining Base	Temp.	Ionization Constant k_a	Ionization Constant k_b	Minimum Molecular Weight
1250 ( )	25	2.6×10	1.2×10^{-1}	33 800 ( )
—	—	—	—	—
—	18	4×10^{7}	1×10^{-1}	45 000 (3)
			1.6×10^{-10} ( ) to 1.6×10^{-1}	
—	—	—	—	—
—	—	—	—	—
—	—	—	—	—
—	—	—	—	—
3000 ( ) 2130 ( )	25	$1\times10^{-}$ ( )	2×10^{10} to $2\times10^{-}$ ( )	12 000(1)
	25	$2.3\times10^{-}$	3.2×10	28 000(3)
—	25	1×10^{-8}	2.5×10^{1}	28 000( )
—	25	1.6×10^{8}	$5.0\times10^{-}$ ( )	60 000(3)
—	—	—	—	—
—	—	—	—	—
1st stage 3290 ( ) 2nd 2060	25	$k_1=2\times10^{-}$ (1) $k_a=5\times10^{-}$	5×10^{-1} ( )	10 300
1700 ( ) 1790 (3)	25	$2\times10^{-}$	6×10 ( )	
—	—	—	—	—
—	25	2.2×10^{5}	$2.9\times10^{-}$	—
—	—	—	—	—
—	—	—	—	—
1st stage = 2100( ) 2nd = 546	25	2.7×10^{5}	2.3×10	190 000
75	25	1.8×10^{-1}	$2.7\times10^{-}$ ( )	75
		1.2×10	1.9×10 ( )	
186	25	1.4×10	$k_{b_1}=1\times10^{-}$ (3) $k_b=1\times10$	186
76	25	$k_a=1.5\times10^{-}$ $k=1.3\times10^{0}$		133
—	18–25	—	1.7×10^{-5} (3) to 1.8×10	
—	18–25	$1.8\times10^{-}$ (3)	—	
—	18	$k_{a_1}=3.0\times10^{-}$ ( )	—	
	25	$k_a=6\times10^{1}$ ( )		

(20) Hitchcock 1922
( ) Jordan Lloyd and Mayes 1922
( ) Greenberg and Schmidt 1923–4
(3) Hitchcock 1923/4
( ) Hitchcock 1923/4
(5) Porter 1921
(26) Meunier Chambard and Jamet 1925
( ) Michaelis and Pechstein 1910
( ) Cohn and Berggren 1924/5
( ) Winkelblech 1901
(30) Harri 1924
(1) Harris 1925
( ) Noyes Kato and Sosman 1910
(33) Wakler and Cormack 1900
( ) McCoy 1903

Concentrated Solutions of Acids and Alkalis

Although on either side of the iso electric point proteins combine with acids and alkalis through their free amino or carboxylic groups respectively there is evidence that at a higher concentration of acid or alkali there is a greater fixation of electrolyte than can be accounted for on this theory Jordan Lloyd and Mayes (1921) find that at *p*H 1 7 ([H] =

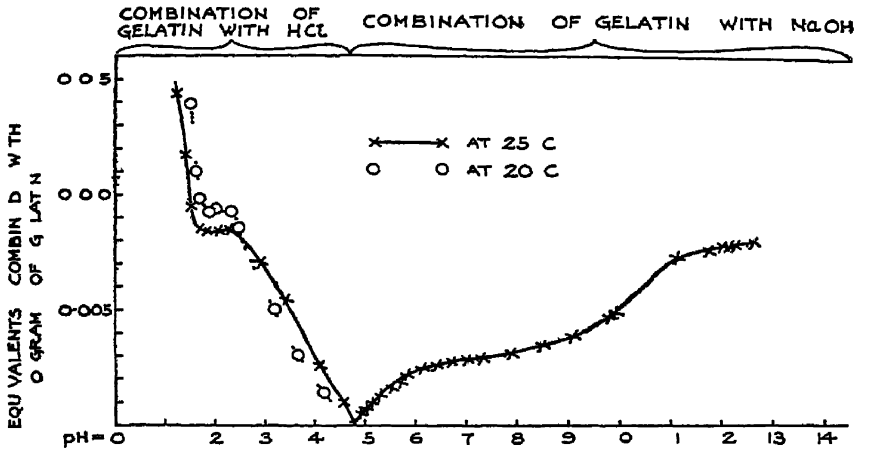


FIG 18 —The influence of hydrogen ion concentration on the combination of gelatin with hydrochloric ac d and sodium hydroxide Curve at 20 from the experimental results of Jordan Lloyd and Mayes curve at 25 from those of Atkin and Douglas
(From Jordan Lloyd and Mayes *Proceedings of the Royal Society B* 1922 nd Atkin and Douglas *Journal of the Society of Leather Trades Chemists* 1924)

0 02) or less there is an increased fixation of acid by the protein and at *p*H 11 ([OH] = 0 003) or greater there is a very greatly increased fixation of base Atkin and Douglas (1924) also find a very great increase in acid or base fixation at *p*H 1 7 and *p*H about 10 respectively The titration curve of gelatin plotted from the values given by Jordan Lloyd and Mayes and by Atkin and Douglas are shown in Fig 18 Since hydrolysis of the protein by acid or base in these strong solutions does not materially affect the acid or base binding power (Robertson 1918 Jordan Lloyd and Mayes 1921)

the possibility of further fixation of electrolytes at the peptide link or other linkages has to be taken into account Robertson (1924) has examined the acid and base binding power of casein and gelatin at *p*H 2 0 8 2 and 10 5 and finds that except with gelatin at 10 5 there is actually a decrease in the ratio

$$\frac{\text{equivalents of acid or base bound}}{\text{equivalents of free amino } N}$$

after hydrolysis with trypsin

The Reactions of Proteins and Acids near the Iso electric Point The Hermaphrodite Ions of Proteins (Zwitterions)

Pauli and Modern (1925) have recently studied the fixation of the ions of hydrochloric acid by gelatin and serum albumin at acid concentrations of less than 0 01 *N* They find a much greater fixation of the hydrogen than the chlorine ion at all concentrations within the limits of their experiments and consider with the majority of other workers that the protein forms an ionisable hydrochloride with the acid They do not however consider that this salt is fully ionised at all reactions but that the ionisation increases with increasing hydrogen ion activity Taking α as the degree of ionisation of the gelatin chloride and equal to $\frac{C_H - C_{Cl}}{C_H}$ where C_H and C_{Cl} are the concentrations of the hydrogen and chlorine ions they obtain the following values —

Concentration of HCl as normality	1×10	3×10	5×10	1×10^{-3}	5×10^{-3}	1×10
(per cent)	9 64	47 3	60 17	67 64	67 17	57 42

By means of conductivity measurements they now reckon

(1) The total conductivity found by direct measurement

(2) The conductivity due to the water
(3) The conductivity due to free hydrochloric acid
(4) The conductivity due to ionised gelatin chloride the conductivity of all protein ions being taken as 50 reciprocal ohms (Adolf 1923)
(5) The conductivity of gelatin as an acid

After subtracting (2) (3) (4) and (5) from (1) there remains a residual conductivity which is ascribed to the presence of what may be called the hermaphrodite ions (zwitterions) of the protein—$^{+}HN_3$ R COO^{-} Ions of this type have been postulated by Bjerrum (1923) for amino acids They carry both a positive and a negative charge are strongly bi polar and tend to associate in pairs Under the influence not of the hydrochloric acid but of the carbon dioxide of the air (Pauli and Schon 1924) these associated herma phrodite ions separate as positive and negative protein ions —

$$2\ {}^{+}NH_3\ R\ COO^{-} + \tfrac{1}{2}(H_2CO_3) \rightarrow NH_3(\tfrac{1}{2}CO_3)\ R\ COO^{-} + COOH\ R\ NH_3^{+}$$

The residual conductivity of gelatin due to positive and negative protein ions is said to vanish at a hydrochloric acid concentration of 0 0008 *N* The residual conductivity of serum albumin however due to positive and negative protein ions called into being both by carbonic acid and hydrochloric acid persists in all concentrations of acid examined *i e* up to 0 01 *N* hydrochloric acid

The existence of hermaphrodite or zwitterions of proteins is a possibility on which further experimental evidence is greatly needed They form however an interesting basis to account for the interactions of the proteins and the neutral salts at reactions near the iso electric point (see Pauli and Schon 1924)

Strong and Weak Acids and Bases

The relationship of various proteins to hydrochloric acid and to sodium hydroxide has been dealt with at length since

both these electrolytes are strongly ionised and monovalent and therefore the equations by which the general relations can be expressed appear in the simplest form These may be summed up as follows —

(1) The amount of acid (or base) combined with a protein (*i e* the amount of protein salt present) depends directly on the hydrogen ion concentration of the solution

(2) The reaction (*p*H) at which the protein is fully con verted into the salt form depends on the iso electric point of the protein and the value of its ionisation constants

It is obviously important to see if these two generalisations can be extended to apply to systems containing other acids Pauli and Hirschfeld (1914) examined the combination of gelatin and serum albumin with hydrochloric sulphuric and acetic acid respectively The combination of the weak acid acetic acid was calculated from the equation

$$C_{CH_3COO} = \frac{K\,(n - C_H) - (C_H)^2}{K + C_H}$$

where n = the total concentration of the acid C_H = the concentration of the hydrogen ion measured by the hydrogen electrode K the ionisation constant of acetic acid at the temperature of the experiment and C_{CH_3COO} the acid bound by the protein Pauli and Hirschfeld considered that for the same concentration of acid the amount of acid bound was less for a weak acid than for a strong one For the same concentration of hydrogen ion they considered that the amount of acid bound was greater for a weak acid than a strong one It can be seen however from Table XVIII of Pauli and Hirschfeld s paper that the hydrogen ion con centration which they are considering is the one produced by the acid in the absence of the protein and it was shown later by Loeb (1918/19 1920/1 1922) that if acid bound were plotted against the final equilibrium concentration of hydrogen ion then the formation of protein salt was con trolled only by the *p*H of the system provided that the

ionisation constant of the acid did not fall below a certain minimum value The latter point is important and follows readily from a consideration of the properties of proteins taken in conjunction with those of a tri basic acid such as phosphoric acid In Fig 19 is shown the titration curve of phosphoric acid by deci normal potash It can be seen that from *p*H 0 to 4 5 only H_3PO_4 and KH_2PO_4 are present in the system From *p*H 4 5 to 9 5 KH_2PO_4 and K_2HPO_4 are

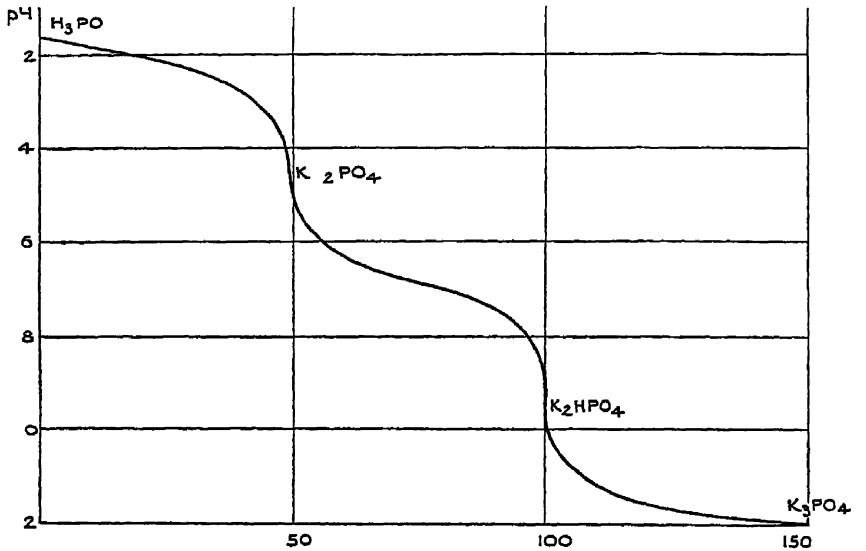


Fig 19 —50 c c N/10 H P O titrated with N/10 KOH
(From Clark Determ nation of Hydrogen Ions)

present and from *p*H 9 5 onwards K_3PO_4 appears Now most proteins are very weak bases having K_b of the order $1—10 \times 10^{-11}$ and iso electric points between *p*H 4 7 and 7 Their basic function therefore will be limited to the *p*H range where the only acid present is $H^+ + H_2PO_4$ and the salts they form with phosphoric acid therefore will be mainly of the type $R–NH_2H\,H_2PO_4$ The ionisation of phosphoric acid may be represented to occur in three stages (Abbot and Bray 1909) —

(1) $H_3PO_4 \rightleftarrows H + H PO_4$ $l = 1\,1 \times 10^{-2}$

(2) $H_2PO_4 \rightleftarrows H + HPO_4$ $k = 2\,0 \times 10^{-7}$

(3) $HPO_4 \rightleftarrows H + PO_1$ $l = 3\,6 \times 10^{-13}$

By analogy therefore it might be anticipated that for many proteins salt formation can only occur with an acid having an ionisation constant $> 1 \times 10^{-7}$ from which it

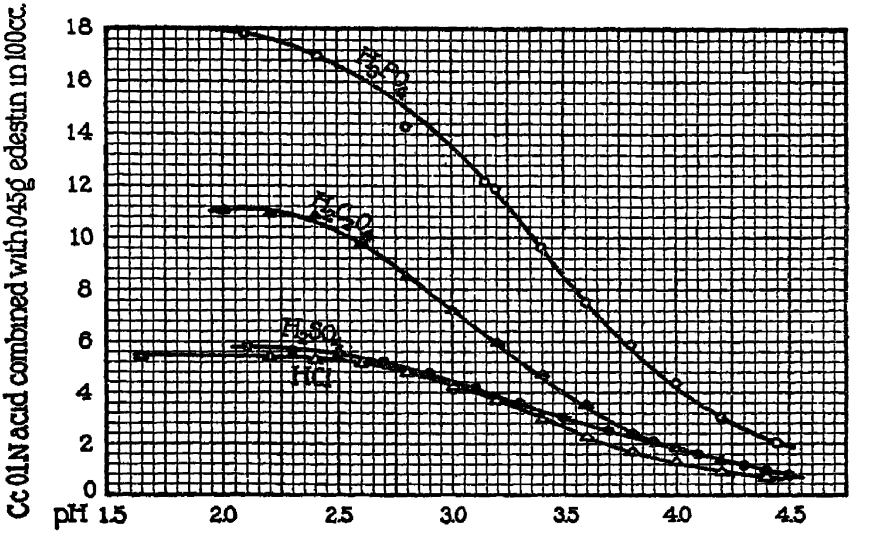


FIG 20 —Amounts of 0 1 N acid combined w th 0 4ɔ gm edestin in 100 c c at 25 Values of hydrochl r sulphur c and oxal c ac ds obta ned by difference between titration curves with and without protein Values for phosphor c acid btained by calculat on

(From H tchcock *Journal of General Physiology* 192ᵖ)

will follow that organic acids as a class will behave towards most proteins as if they were monobasic or in other words they will combine with proteins in molecular proportions

This generalisation was first placed on record as an experimental observation by Hardy (1905) in his classical paper on the globulins It was confirmed by Loeb (1922) and also by Hitchcock (1922) In Fig 20 is reproduced the curve of combination of edestin with hydrochloric sulphuric oxalic and phosphoric acids respectively taken from Hitchcock s

paper The amount of acid combined with the protein is expressed as cubic centimetres of deci normal acid It can be seen from the figure that at any value of *p*H in the system the number of cubic centimetres of acid combined with the protein varies directly with the basicity of the acid *i e* the ratio hydrochloric acid oxalic phosphoric is 1 2 3 in other words the combination is in molecular proportions all the acids behaving like monobasic acids The strong dibasic acid sulphuric acid does not conform to this generalisation its combination with edestin compared to hydrochloric acid being in equivalent and not in molecular proportions This apparent exception proves the general rule for both the hydrogen ions of sulphuric acid have a very high ionisation constant and both therefore are available for salt formation even with bases as weak as the proteins It must be remembered however in generalising on the combination of proteins and acids that what holds true for the nearly neutral proteins such as albumins globulins casein and gelatin will not necessarily hold in the case of the strongly basic histones and protamines These have received very little attention up to the present but it is to be anticipated that their K_b value may possibly rise to the order of 10^{-5} and their iso electric point move over to the alkaline side of absolute neutrality They will probably not only form salts of the type R NH H H_2PO_4 but also of the type

$$\left.\begin{matrix} R\ NH_2H \\ R\ NH_2H \end{matrix}\right\rangle HPO_4$$

The combination of proteins with different bases has not been studied in such detail as their combination with acids Direct experiments on the influence of the ionisation constant of the base are lacking though Handovsky s (1910) work on the viscosity of albumin solutions in the presence of equal concentrations of ammonia di ethylamine tetra ethyl ammonium hydroxide and sodium hydroxide suggest by analogy that at any given total concentration of base the

extent of combination is a function of the ionisation constant At equal *p*H values proteins combine with strong bases in equivalent proportions (Robertson 1918 Loeb 1920/21 1922 Adolf 1923 Hitchcock 1922) Hitchcock s titration

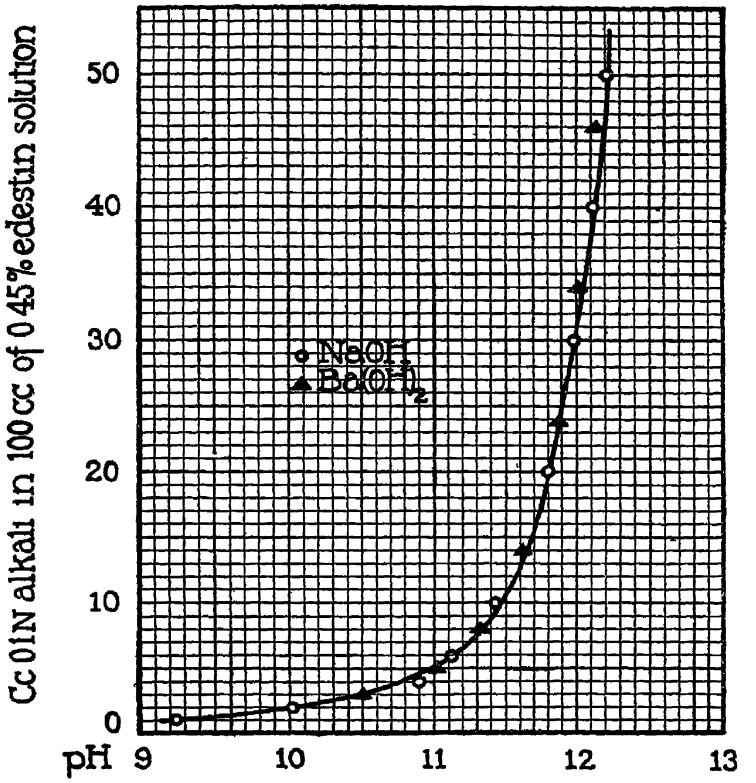


Fig 21 —Titration curve of 0 45 per cent edestin with 0 1 N sodium hydroxide and 0 1 N barium hydroxide Temperature = 25
(From Hitchcock *Journal of General Physiology* 1922)

of edestin by the hydroxides of sodium and barium is shown in Fig 21 It can be seen that the same amount of base is required to bring the solution to the same *p*H value in both cases

Combination of Protein with Acids and Bases in the Presence of Salts

There has been a considerable amount of experimental work published on the possibility of salts combining with proteins

some of which will be discussed later While any actual combination between neutral salt and protein has not yet been satisfactorily proved it seems now quite clear that the presence of salts influences combination of the proteins with acids or alkalis Csapo (1925) gives the following figures for 0 9 per cent gelatin and $N/100$ hydrochloric acid in the presence of sodium salts at a concentration of about $N/3$

Anion	Initial pH without gelatin	Final pH with gelatin	H^+ bound
—	1 98	2 30	$5\ 42 \times 10^{-3}$
Cl	1 98	2 39	$6\ 33 \times 10^{-3}$
I	2 00	2 45	$6\ 47 \times 10^{-3}$
NO_3	1 96	2 34	$6\ 21 \times 10^{-3}$
SO_4	2 01	2 12	$2\ 11 \times 10^{-3}$

Gerngross and Lowe (1922) also find increased fixation of alkali by hide powder on the addition of neutral salts

Determination of the Iso electric Points of Proteins by Titration Curves

It follows from what has been said above that the iso electric points of proteins can be determined by ascertaining the reaction at which the protein does not alter the pH of an unbuffered solution or does not combine with either hydrogen or hydroxyl ions This method has been used by Meunier Chambard and Jamet (1925) for collagen by Patten and Kellem (1920) and in a modified form using acid and basic dyes as indicators by Thomas and Kelly (1922) With pure proteins the method is very reliable but in the presence of electrolytic impurities it is liable to error (see Slator Price 1923 Rawling and Clark 1921)

Reactions with Salts in the Presence of Acid or Alkali

Loeb (1918/19 1922) has shown that in the presence of acids or alkalis the resulting protein salt can interact with any other salt present in the system The nature of the

interaction is decided by the reaction of the solution in relation to the iso electric point On the alkaline side the protein acts as an additional acid and combines with the base of the added salt on the acid side with the acid radicle

It has long been known that gelatin in alkaline solution combines with silver to form gelatin silver compounds which blacken on standing in the light Loeb showed that these compounds are formed on the alkaline side of the iso electric point only and that the amount of silver bound was a function of the reaction of the solution He added powdered gelatin to a solution of silver nitrate adjusted a series of reactions with nitric acid between *p*H 6 and 3 and allowed his experimental tubes to stand He then drained away the supernatant fluid and washed the gelatin powder with several changes of ice cold distilled water The experimental samples were melted and made up to a fixed volume or analysed In tubes more alkaline than *p*H 4 7 the gelatin blackened slowly in the light in tubes more acid there was no change

In the systems more alkaline than *p*H 4 7 containing negatively charged gelatin (probably partly as a calcium salt) and silver nitrate there are present the positive ions silver and two negative ions nitrate ion and gelatinate ion It is therefore not surprising that some interchange should be effected leading to the formation of some silver gelatinate This salt being non diffusible is retained with the gelatin whilst the excess of silver nitrate is washed away On the acid side of the iso electric point a different system occurs The gelatin is now positively charged *i e* it is functioning as a base Hence in the system gelatin nitric acid silver nitrate there are two bases gelatin and silver and one acid nitric acid Hence there can be no reaction between the gelatin and the silver and in washing the gelatin powder all the silver is removed Loeb showed that all positive ions behave like silver *i e* they combine with proteins on the alkaline side only of the iso electric point Conversely all

negative ions combine with proteins on the acid side of the iso electric point only for instance if gelatin powder is mixed with sodium bromide and the reaction adjusted after washing it is found that bromine is retained in all the experi mental tubes which had a reaction more acid than *p*H 4 7 but not in tubes more alkaline

The examples given above are typical of the behaviour of proteins towards acids bases and neutral salts It would perhaps be useful to add a note on the use of such expressions as gelatin chloride and sodium gelatinate These phrases are convenient short expressions particularly useful for writing on diagrams and distinguishing curves several of which may be plotted together on one figure Wherever they are used however it must always be borne in mind that they are not names of definite chemical entities There are probably at least ten gelatin hydrochlorides all of which may be present together and no one of which has yet been isolated or probably ever will be isolated as a single individual and at least four sodium gelatinates of which the same is true The need for a brief terminology justifies the use of these terms but the bestowal of a name does not create a definite substance where none exists

Attention must also be called to the fact that a second theory exists that the reduction of free acidity or free alkalinity which takes place if protein is added to a system is due not to chemical combination but to adsorption (see especially Izaguire 1923 and Gerngross and Lowe 1922) Chemical combination is now attributed to electronic interchange between the combining groups adsorption to the attraction due to residual fields of force It is very doubtful in the case of a large complicated system like a protein whether it will ever be possible to draw a sharp line between the two classes of compounds Moreover it is now recognised that surface adsorption is affected by the chemical nature of both adsorb ing surfaces (Hardy 1913 Hardy and Doubleday 1922)

The theory that proteins form ionisable salts with acids and

bases rather than adsorption compounds has in its favour the fact that it is a mental tool easily available to most chemists and that inductively and deductively it fits in very fairly with experimental observations Like most scientific theories it should be recognised that it only holds between certain limits

Protein Buffers

The weakly acidic and weakly basic character of proteins and protein derivatives gives to their aqueous solutions the properties characteristic of buffers The feature of a buffer solution is that large changes in a total acid or base content are accompanied only by small changes in the reaction of the solution Buffered solutions have many advantages when it is desired to keep the reaction of a system steady as for instance frequently happens in the cultivation of bacteria The buffer action of a solution of a pure protein could very easily be reckoned but the buffer action of bacterial media containing peptones and amino acids in unknown proportions can only be found by experiment As a result in adjusting the reaction of such media it is important that the *p*H desired should be determined directly Many micro organisms are very sensitive to hydrogen ion concentrations and the old method of adjusting reactions by titrating to some arbitrary end point and then adding an empirical fraction of the titre is one which can only be used when there is certain knowledge that the buffer content of the medium is the same from one batch to another

It is difficult to estimate to what extent the buffer action is of importance in the living animal or plant body It has been shown for instance that the plasma proteins take part in maintaining a steady *p*H value in the blood and the muscle proteins also interact with the lactic acid produced during the course of muscular work and so prevent the hydrogen ion activity in the fibre from reaching too high a value In living cells the reaction is never very far removed from the iso electric points of the cell proteins and it seems probable

that the very labile system resulting with its possibilities of readily changing from uncharged slightly soluble protein to electrically active highly soluble protein salt is one which is particularly well adapted for maintaining the equilibrium of the body fluids

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CHAPTER IX

THE PROPERTIES OF PROTEIN SOLUTIONS

Osmotic Pressures and Membrane Equilibrium—Membrane Potentials—Migration of Protein Ions—The State of Proteins in Solution—Viscosity—The Behaviour of Proteins on Drying—Distribution of Water in Blood and Tissues—The Physical Chemistry of Bread making

Osmotic Pressures and Membrane Equilibrium

THE two properties of solutions which give the clearest vision of the relations of solvent and solute are the osmotic pressure and the viscosity The former is a measure of the number of particles of solute which are moving freely through the solvent by virtue of their own kinetic energy the latter is an indication of the relative volume occupied by these particles in the solution Osmotic pressures in aqueous solutions can be measured directly by counter balancing them against the hydrostatic pressure of a column of water drawn into the solution through a boundary permeable to the water but impermeable to the enclosed solute The osmotic pressure of protein solutions has been examined by a method based on this fundamental idea Proteins are as seen in the last chapter amphoteric in character At the iso electric point they dissolve as the electrically neutral ampholyte At other reactions they are mainly present as the positive or negative ions of the protein salts The low values of K_a and K_b show that in aqueous solutions protein salts will be very largely hydrolised with a reversion of a large proportion of the protein to its condition of un ionised acid or base and a consequent reduction of the number of protein ions in the solution Protein ions (disregarding for the time being the hæmaphro

dite ions) can only exist in solution in the presence of an excess of acid or base and it is therefore obvious that the properties of proteins in solution will be largely affected by the hydrogen ion concentration

The actual osmotic pressure of a protein solution consists generally of two fractions—the one due to the dissolved protein and protein salts and the other due to the excess of electrolyte present The early history of the experimental work on this subject is the story of many attempts to separate these two factors The method employed was to measure the osmotic pressure of the protein solutions in osmometers formed not from semi permeable membranes such as are used for solutions of crystalloids but from permeable membranes such as parchment paper or collodion The principle underlying the method was that the electrolytes present might be expected to come to an equal distribution on both sides of the membrane any pressure recorded in the osmometer being due therefore to the colloidal proteins In this way Starling (1896 1899) demonstrated that the proteins of serum contribute a share to the osmotic pressure of the blood and Moore and Roaf (1907) obtained experimental evidence that gelatin in solution exerts a measurable osmotic pressure Measurements of this type made between the iso electric point of the protein and the neutral point of water give results which later work has shown are close to the true values the early work of Reid (1904 1905) for instance on the osmotic pressure of hæmoglobin having recently been confirmed by Adair (1925) When however the method was extended by Lillie (1907/8) and other workers to investigate the osmotic pressure of proteins at varying hydrogen ion concentration a new source of error crept in which was not at first appreciated This error arises from the fact that an electrically charged colloidal particle has a measurable influence on the distribution of diffusible electrolytes across a membrane and hence osmotic pressure readings at all reactions other than the iso electric point are not a direct measure of the osmotic

pressure of the protein salts in solution but involve a factor due to the unequal distribution of the diffusible ions The factors controlling the distribution of diffusible and non diffusible ions across a membrane were worked out on a thermodynamical basis by Donnan (1911) and the type of equilibrium reached in such systems has now become known as Donnan s membrane equilibrium The theory of mem brane equilibrium is based on the following consideration namely that if a non diffusible ion is present in a system bounded by a membrane it will exert an electrostatic repul sion on the diffusible ions of the same sign leading to the expulsion of these to the opposite side of the membrane—*i e* in a system containing a non diffusible positive ion all the non diffusible positive ions will be on one side of the mem brane and the majority of the diffusible positive ions on the other the actual number of positive diffusible ions on the two sides of the membrane depending on the relative concen trations of non diffusible and diffusible ions in the system The distribution of the negative ions takes place in such a way as to lead to electric neutrality on either side of the membrane Hence it follows that in any system containing a colloidal ion and bounded by a permeable membrane there is a force which checks the diffusion of electrolytes into the system This force is directly proportional to the concen tration of the non diffusible ion

An application of the principle of membrane equilibrium to protein solutions leads to the following conclusions —

(1) At the iso electric point the distribution of electrolytes across a membrane will not be affected by proteins in solution and the osmotic pressure recorded will be due entirely to the protein

(2) At reactions removed from the iso electric point the proteins in solution combine with the acid (or base) present to form ionisable colloidal salts The positively charged protein ions in an acid system then repel the free hydrogen ions (and any other positive ions which may be present) across the

membrane—*i e* in a system consisting of a protein dissolved in dilute acid and bounded by a membrane equilibrium will not be reached until more than half of the free acid has been expelled across the membrane

For instance in a system consisting of gelatin and hydrochloric acid dissolved in water there will be present electrically neutral gelatin particles positive gelatin ions chlorine ions derived from the ionised gelatin salts hydrogen ions chlorine ions derived from the ionised acid and un ionised hydrochloric acid Such a system enclosed in a permeable membrane and in contact with water may be represented as

$$\underset{a}{[G]} + \underset{b}{[G^+]} + \underset{z}{[Cl^-]} + \underset{y}{[H^+]} + \underset{y}{[Cl^-]} + \underset{c}{[HCl]} \;\Big|\; \underset{x}{[H^+]} + \underset{x}{[Cl^-]} + \underset{c}{[HCl]}$$

where a b z y x represent concentrations It should be noted that b is not equal to z since the gelatin ion is a multivalent ion of unknown and variable valency Further in dilute solutions c will be infinitely small and may be neglected

For thermodynamic equilibrium the product of positive and negative ions on one side of the membrane must equal the product of positive and negative ions on the other

$$(b + y)(\ + y) = x$$

Now in the absence of gelatin salts *i e* when b and z both equal 0

$$y = x$$

and the distribution of the acid will be equal on both sides of the membrane but for all positive values of b and z

$$y < a$$

and it is obvious that the greater the values of b and z the greater will be the inequality between x and y Therefore the concentration of the acid outside the membrane is greater than the concentration inside by an amount which is directly determined by the concentration of the ionised gelatin salt This inequality was first demonstrated for protein solutions

by Proctor (1914) and afterwards confirmed by other workers including Loeb It follows therefore in measuring the osmotic pressure of these solutions that the distribution of the water across the osmometer membrane will be affected not only by the ions inside the membrane but also by those without in other words the pressure recorded by the osmometer is the pressure of the protein salt *plus* free acid inside the membrane *minus* the pressure of the acid outside The pressure of 1 mol of any ion or molecule (or molecular aggregate) is a constant quantity for the same conditions of temperature and may be denoted by K At 20 K is approximately $2\ 5 \times 10^5$ mm of water

The pressure of a gelatin acid solution at 20 recorded in an osmometer with a permeable membrane should therefore be equal to —

$$[a + b + z + 2y - 2a]\ 2\ 5 \times 10^5 \text{ mm of water}$$

In considering the osmotic pressure of proteins in solution there are therefore two entirely different problems involved The first is the investigation of the osmotic pressure due to the dissolved protein itself *i e* the evaluation of $[a + b]$ K This was the problem approached by Starling Moore Reid Roaf Adair and others A number of values for different proteins have been obtained near the iso electric points but a thorough study of the variation of $[a + b]$ K with different external conditions still awaits investigation

In the case of hæmoglobin the iso electric point of which is at *p*H 6 8 Adair (1925) has shown that the osmotic pressure in distilled water *i e* at the iso electric point is a constant low quantity varying directly with the concentration of dissolved protein and suggesting a molecular weight of about 67 000 He finds no suggestion of molecular aggregation in solutions as concentrated as 20 per cent Higher osmotic pressures suggesting lower molecular weights he attributes to the presence of acid (carbon dioxide) or alkali in the solution Neutral salts have no influence on the

osmotic pressure recorded in neutral solution With hæmoglobin therefore under conditions when $b = 0$ the value of $[a + b]K$ depends directly on the concentration of the protein Egg albumin in which the osmotic pressure determinations give a molecular weight of 34 000 (Sørensen 1917) a value coinciding with the value deduced from chemical evidence also dissolves like hæmoglobin *i e* only in the molecular condition An investigation of the osmotic pressure of globulin gelatin or casein at their iso electric points and in the presence of salts might throw valuable light on their state of aggregation in solution

The second problem in considering the osmotic pressure of protein solutions is the investigation of the variation of the whole expression

$$[a + b + z + 2y - 2x]K$$

according to the external conditions This problem which is of the greatest physiological importance since it is essentially the study of the distribution of water in living tissues has been investigated in great detail by Loeb and his co workers To simplify the investigation they have neglected the factor $[a + b]K$ and considered only the variation of the osmotic pressure and of the expression $[+ 2y - 2x]K$ according to the variation of y and x

With the case of proteins in acid solutions x is the hydrogen ion concentration outside the membrane y the hydrogen ion concentration inside the membrane z the concentration of the negative ion of the protein salt can be calculated from the equations —

$$z = \frac{(x - y)\ (x - y)}{y} \text{ for monobasic acids}$$

$$z = \frac{x^3 - y^3}{y^2} \text{ for dibasic acids}$$

These two equations are derived directly from the relation of thermodynamic equality referred to above where

$$y\ (y + z) = x^2 \text{ for monobasic acids}$$

$$y^2 \left(\frac{y + z}{2}\right) = x \left(\frac{x}{2}\right) \text{ for dibasic acids}$$

The justification for the somewhat arbitrary neglect of a and b the pressures due to the protein is that owing to the magnitude of the protein molecule solutions containing from 1 to 5 per cent of protein are in terms of equivalent concentration very dilute say of the order 10^{-3} and in terms of concentration of kinetically independent particles possibly

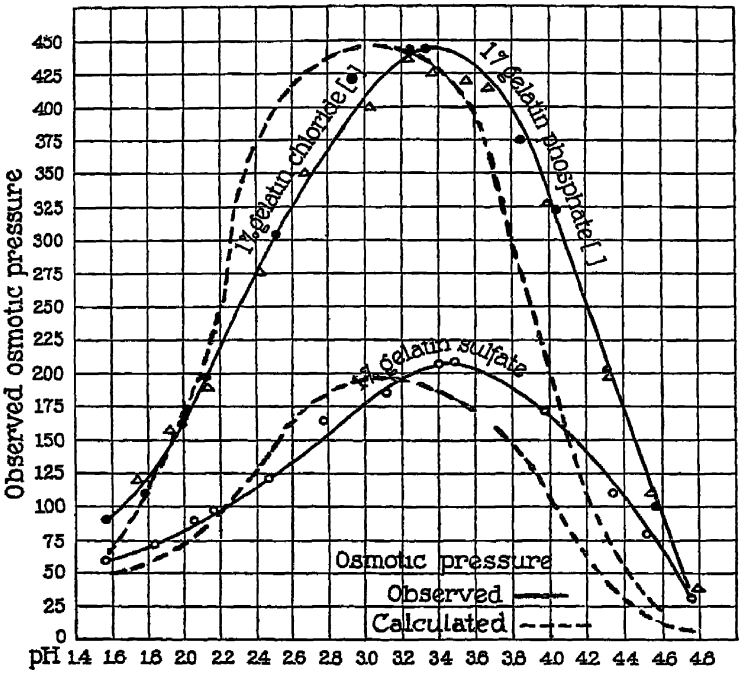

FIG 22.—Osmotic pressure in millimetres of water of a 1 per cent gelatin solution. Abscissæ give the hydrogen ion concentration of the gelatin solution at equilibrium. Temperature = 24.
(From Loeb *Journal of General Physiology* 1920–21.)

even less say 10^{-4} or 10^{-5}. Loeb and his co-workers have made a detailed study of the osmotic pressure of protein solutions in the presence of free acid, free alkali and of various neutral salts. They have demonstrated that the distribution of the diffusible ions which takes place in accordance with Donnan's theory has a very important influence on the osmotic pressure recorded: the low values at the iso-electric

point the rapid rise on either side to acid and alkaline maxima and the fall with further increase of concentration the reduction of osmotic pressure on the addition of neutral salts are all accounted for by the theory of the formation of diffusible ions on the two sides of the membrane Loeb's experimental curve for the osmotic pressure of gelatin solutions in the presence of hydrochloric and sulphuric acid are shown in Fig 22 together with the two curves calculated for a monobasic and a dibasic acid by means of the equation

$$(z + 2y - 2x)\,2\,5 \times 10^5 = \text{osmotic pressure}$$

The parallelism between the observed and calculated curves is very striking but the definite discrepancy between them suggests that the osmotic pressure due to the protein particles $(a + b)$ also has a value too great to be neglected

The osmotic pressure is plotted as ordinate and $-\log y$ or the pH of the internal solution as abscissa It can be seen that in both systems the osmotic pressure is lowest at or near the iso electric point (pH 4 7) rises to a maximum and falls again The graphs also show that the valency of the electrolyte has a definite effect on the value of the maxima the monobasic acids resulting in osmotic pressure approximately twice as high as the strong dibasic acid at the same pH The same valency rule holds for the osmotic pressure in alkaline solutions The maximum osmotic pressure of protein solutions is calculated by Loeb after making allowances for certain corrections to be at a pH of 3 2 to 3 4 in acid solutions The minimum osmotic pressure is found at the iso electric point

Membrane Potentials

An interesting corollary from the study of the osmotic pressure of protein solutions and the distribution of ions across a membrane is that whenever an ionised protein is enclosed in a membrane there will be found a charge at the surface This potential arises from the formation of a liquid junction of two unequal concentrations of the same electro

lyte Loeb (1920–21 1921–22) has measured the charge on the membrane under varying external conditions It is minimal (< 10 millivolts) at the iso electric point rises to a maximum on either side and then falls again In acid solutions the protein solution is positive to the external

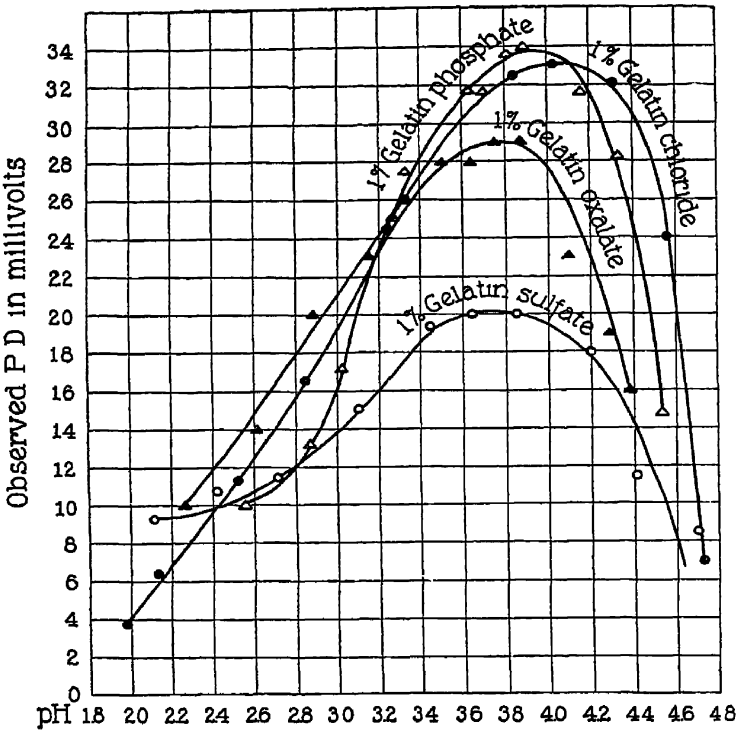

FIG 23 —Curve showing the variation of the potential on the surface of an osmometer membrane with the hydro en ion concentiation of the internal gelatin solution n the presence of different acids Temperatuie = 24

(From Loeb *Journal of General Physiology* 1920–21)

solution in alkaline solutions negative Experimental curves for the variation of the potential of the membranc (P D) are shown in Fig 23 The potential varies with the hydrogen ion concentration and the valency of the anion of the acid present sulphuric acid with a divalent anion giving only half the potential of hydrochloric acid Phosphoric acid

behaves like a monobasic acid and oxalic acid is intermediate Loeb considers that the origin of the charge on the surface of all living cells is due to the formation of a membrane equilibrium at their free surface This is doubtless true for all free cells for example suspensions of bacteria and red blood cells but the cells of a multicellular organism *in vivo* are subject to more complicated conditions than *in vitro* since there will be non diffusible ions on both sides of their free surface

Migration of Protein Ions

The charge on protein molecules in solution on either side of the iso electric point is due to the ionisation of protein salts Michaelis and Airila (1921) have determined the velocity of migration of hæmoglobin at various concentrations of the hydrogen ion Their experimental curve is shown in Fig 24 Between *p*H 5 and 9 it corresponds to the curve which would be anticipated for a typical ampholyte with $k = 10^{-7\cdot8}$ and $k_b = 10^{-8\cdot3}$ *i e* there is no movement at *p*H 6 8 the iso electric point and increased speed of movement as the reaction changes from this value to more acid or more alkaline At *p*H 3 7 the hæmoglobin is rapidly transformed into hæmatin The charge on the hæmoglobin can therefore be attributed entirely to the ionisation of hæmoglobin salts Svedberg and Jette (1923) have studied the velocity of migration of egg albumin and Adolf (1923) the migration of serum globulin

The increased speed of migration on either side of the iso electric point may be regarded in a protein such as hæmoglobin as the result of an increasing charge due to increased ionisation on a particle of constant mass (the hæmoglobin molecule) With proteins which form molecular aggregates the potential difference between the particle and liquid in which it is lying is influenced by the distribution of diffusible ions across the surface of the particle Loeb (1922) has measured the potential difference on gelatin particles in

suspension He finds that at the iso electric point there is minimal or no charge on either side of this point there is a

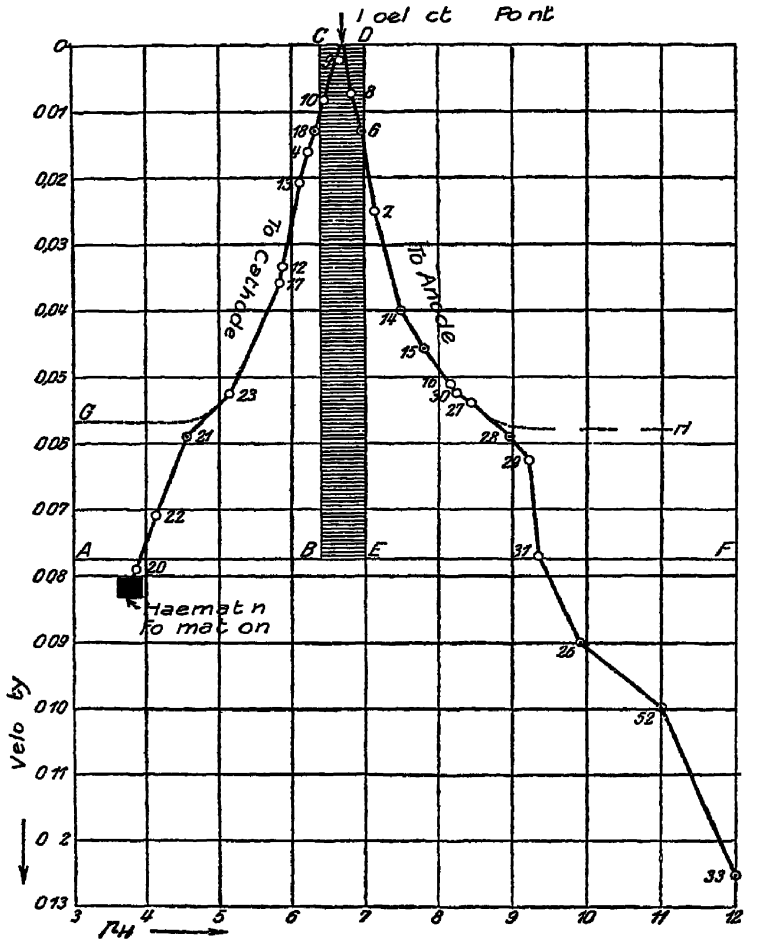

FIG 24 —The migration of hæmoglobin at varying hydrogen ion concentra tions The dotted line GH is the curve calculated for a univalent ampholyte in which $K = 1\,0^{-8}$ $K = 10^{-3}$
(From Michaelis and Airila *Biochemische Zeitschrift* 1921)

charge positive in acid and negative in alkali that rises to a maximum and then declines He used particles large enough to be visible to the naked eye in his measurements of potential difference at varying *p*H but his arguments apply

equally to particles which are sufficiently small to be in colloidal solution and he considers that in the case of gelatin the charge on the colloidal micellæ is controlled by a membrane equilibrium That some proteins do form aggregates in solution can be deduced from the values obtained both from osmotic pressure measurements and viscosity measurements These aggregates which occlude water have a varying charge and a varying mass

The State of Proteins in Solution

Osmotic pressure determinations with certain other evidence which will be given later prove that the albumins and the hæmoglobins dissolve in water as single molecules Both these classes of proteins are easily obtained in the crystalline condition They therefore resemble soluble crystalloids in their behaviour towards water With gelatin however striking differences appear Biltz Bugge and Mehler (1916) determining the molecular weight of gelatin from its osmotic pressure obtained values varying from 5 500 to 31 000 while Smith (1921) obtained 96 000 Chemical evidence suggests that the gelatin molecule has a weight of the order of 10 300 (Jordan Lloyd 1920) and Cohn Hendry and Prentiss (1925) after a recent review of the literature have confirmed this figure It therefore follows that although gelatin can exist in water like a crystalloid as solutions of single molecules to account for an apparent molecular weight of 31 000 or 96 000 it must also exist even in quite dilute solutions in the form of molecular aggregates *The tendency to form molecular aggregates associated with the solvent is characteristic of the proteins as a class* It is displayed by all members except apparently hæmoglobin even by the albumins in sufficiently concentrated solutions and is the basis of many of the colloidal characters of protein solutions The tendency to aggregate formation is least marked in the albumins hæmoglobins and probably also the hæmocyanins all of which have been obtained

crystalline and more marked in the gelatins globulins and casein The former class can be obtained in the form of spherical crystals or globuliths (Bradford 1920) and some of the vegetable proteins form good crystals but with the exception of a single specimen of Bence Jones protein (Wilson 1923) the animal globulins have not so far been obtained in the crystalline sta e

Viscosity

The existence of molecular aggregates in solutions of gelatin is shown by studies in the variation of viscosity with external conditions A physico chemical interpretation of viscosity is not so simple as in the case of osmotic pressure Einstein (1906) finds that the viscosity of a solution is the linear function of the relative volume occupied by the solute *i e*

$$\eta = \eta_0 (1 + 2\ 5\varphi)$$

where η = the viscosity of the solution

η_0 = the viscosity of the solvent

and φ = the relative volume occupied by the solute This formula only agrees with experimental findings over very small values of φ *i e* in very dilute solutions Hatschek (1912) gives

$$\eta = \eta_0 (1 + 4\ 5\varphi)$$

for more concentrated solutions and Arrhenius (1917) gives

$$\log \eta - \log \eta_0 = \vartheta\varphi$$

where ϑ is a constant the value of which depends on the system under consideration

It follows from both the Einstein and the Hatschek formulæ that if a solution could exist in which no association occurred between solute and solvent then the viscosity of the solution would increase in proportion to its concentration Actually however the viscosity is always found to increase at a greater rate than the concentration showing that there

is always some association The slope of the viscosity concentration curve is an indication of the extent to which association between solute and solvent has occurred or in other words of the relative volume occupied by the solvent In general the rise in viscosity with increasing concentration is slow in aqueous solutions of crystalloids but very rapid in solutions of proteins The viscosities of solutions in distilled water of sodium chloride cane sugar crystalline egg albumin and pseudoglobulin at varying concentrations may be quoted as examples and are shown in Table VI All viscosities are expressed in terms of the viscosity of water at the temperature of the experiment which is taken as unity Several of the values are read off from smoothed curves

TABLE VI

Viscosity Coefficients of Aqueous Solutions at varying Concentration

Solute	Temp	Concentration						Reference
		0·0	1·0	0	0	10·0	0·0	
NaCl	20	1·00	1·01	—	1·08	1·18	1·50	Hosking
Cane Sugar	20	1·00	1·03	—	1·13	1·32	1·91	
Egg albumin (*p*H near to 5)	25	1·00	1·05	—	1·25	1·60	3·60	Chick and Lubrzynska
Pseudoglobulin at *p*H 6·8	25	1·00	1·1	—	1·5	3·43	38·7	Chick
Gelatin at 4·6	24	1·00	1·8	2·1	—	—	—	Loeb
Gelatin at 4·6	45	1·0	1·2	1·7	—	—	—	Loeb

It is obvious on comparing these figures that in low concentrations ($<$ 10 per cent) egg albumin behaves in solution like the crystalloids Egg albumin in concentrations greater than 10 per cent and pseudoglobulin and gelatin at all concentrations show a much more rapid rate of increase with concentration which leads to the conclusion that the protein is present in association with considerable amounts of water Experimental curves illustrating the relation between

viscosity and concentration for several proteins is reproduced in Fig 25

The viscosity of a fluid is a function of the temperature the value of η for water being 0 013 0 010 and 0 008 at

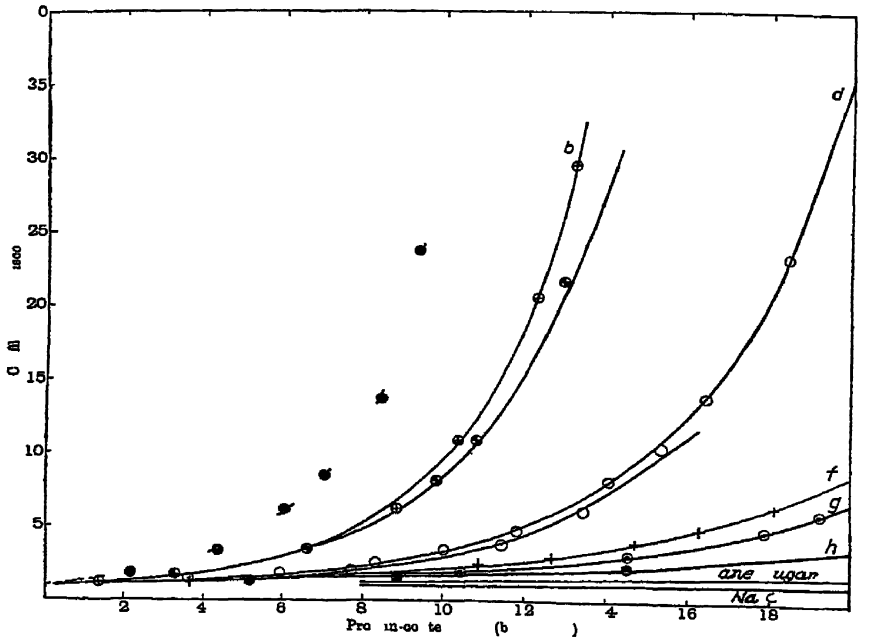

FIG 25.—Variation of viscos ty with concentration Temperature = 25
a = sodium caseinogenate
b = eu lobulin in 5 per cent sodium chloride at *p*H = 5 5–6 0
c = euglobulin in 0 5 to 2 2 per cent sodium chloride at *p*H = 8 0–8 2
d = pseudoglobulin in water at *p*H = 6 8
e = pseudoglobulin in 10 per cent ammonium sulphate at *p*H = 6 8
f = whole serum (horse)
g = serum albumin
h = egg albumin
The curves for cane sugar and sodium chloride have been added
(From Chick *Biochemical Journal* 1914)

10 20 and 30 respectively The influence of dissolved salts is not however affected by change of temperature *i e* the ratio of the viscosity of the salt solution to that of water is the same at all temperatures Here again dilute solutions of albumins behave like crystalloids while strong solutions

of albumins and all solutions of globulins show a marked contrast This is shown in Table VII

TABLE VII

Viscosity Coefficients of Aqueous Solutions at varying Temperatures Figure read off from smoothed curves All measurements are referred to water at the same temperature

Solution	Temperature			Reference
	10	0	30	
Water	1 00	1 00	1 00	
5 per cent NaCl	1 05	1 08	1 09	Hosking
20 per cent NaCl	1 46	1 50	1 52	
7 per cent egg albumin	1 36	1 36	1 34	Chick
28 per cent egg albumin	11 9	10 6	9 7	
10 8 per cent euglobulin in 1 8 per cent NaCl at *p*H 8 0	17 3	11 1	8 1	Chick

It is evident that if the variations in viscosity under different conditions are compared with the variations in osmotic pressure quoted earlier in the chapter evidence from both types of experiment suggests that the albumins in dilute solution form monomolecular solutions similar to crystalloids but that other proteins such as the globulins casein and gelatin form molecular aggregates in association with water molecules and that this tendency to aggregate formation is reduced by rising temperature

Proteins can form solutions in water either in the electrically neutral condition at the iso electric point or as ionisable protein salts at reactions either acid or alkaline to this point The condition in which they dissolve has a considerable effect on the viscosity of the solution Hardy (1905) showed that with globulin the relative viscosity coefficients when dissolved under different conditions had the following values —

		7 6 per nt globulin —		
Water	0 3 *N* $MgSO_4$	0 3 *N* $MgSO_4$	0 0084 *N* HCl	0 0084 *N* N OH
1	1 25	4 7	15 7	69

He considered in agreement with Sackur (1903) that ionised protein had a greater effect on the viscosity of water than un ionised and with Pauli and Samec (1909) attributed this to a

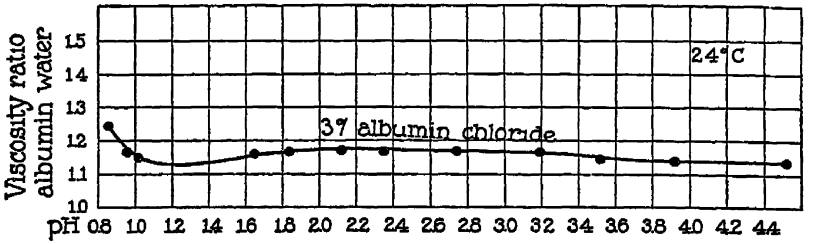

FIG 26.—Influence of hydrogen ion concentration on the viscosity of crystalline egg albumin
(From Loeb *Journal of General Physiology* 1920–21)

greater association between the ionised protein and the water The relation between viscosity and hydrogen ion concentration has recently been investigated by Loeb (1920/1

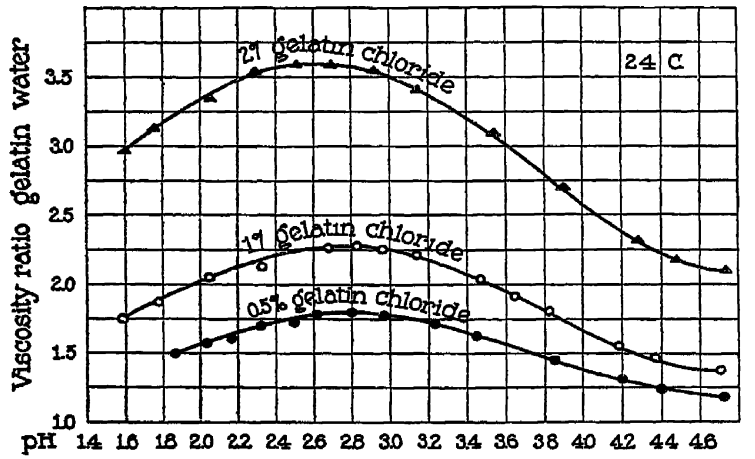

FIG 27.—Influence of hydrogen ion concentration on the viscosity of gelatin
(From Loeb *Journal of General Physiology* 1920/1)

1921/2) who finds the same sharp contrast between the behaviour of albumin on the one hand and casein and gelatin on the other which has already been illustrated in the experiments quoted in Tables VI and VII Loeb's experi

mental results for the variation of the viscosity coefficient of 3 per cent egg albumin (crystalline) and of 2 per cent gelatin solutions in dilute hydrochloric acid are shown in Figs 26 and 27 It can be seen that over a range of reaction from *p*H 4 3 to *p*H 1 0 the viscosity of albumin at 24 hardly varies the viscosity coefficient remaining between 1 1 and 1 2 With gelatin the *p*H value has a very marked effect on viscosity the viscosity coefficient for a 2 per cent solution at 24 rising from 2 1 at *p*H 4 7 to 3 1 at *p*H 2 6 and falling again with increased hydrogen ion activity Casein shows a similar curve to gelatin Curves showing the influence of the negative protein ions present in alkaline solutions on the viscosity coefficients of albumin solutions are unfortunately lacking but the viscosity of casein in alkaline solutions rises from 1 15 at *p*H 6 (sodium hydroxide) to 1 41 at *p*H 11 and then again falls to 1 35 at *p*H 12 (Loeb 1920/1)

Albumin probably passes into solution as single molecules and Loeb points out that from his experiments it follows that the volume occupied by both the electrically neutral molecule and the electrically charged protein ion is of the same order Gelatin however forms molecular aggregates in solution and these aggregates swell on either side of *p*H 4 7 (see Chapter X) water being drawn into the aggregate by osmotic forces due to the establishment of a membrane equilibrium between the aggregate and the surrounding fluid The value of the viscosity coefficient therefore is a function of the volume of the molecular aggregates and this in turn is a function of the hydrogen ion concentration of the solution The coincidence of the point of minimum viscosity in gelatin solution with the iso electric point is shown by the work of Rawling and Clark (1922) Hitchcock (1923) and others

The viscosity of the type of protein solution in which the formation of hydrated aggregates or colloidal micellæ occurs is not constant but varies with time Hardy (1905) pointed out that the viscosity of globulin in alkaline solutions increases on standing whilst in acid solutions it may decrease Loeb

finds that with solutions of casein in hydrochloric acid the viscosity rises with time at reactions between *p*H 3 0 and 2 4 though at greater acid concentration it may fall The classical instance of variation of viscosity with time occurs however with gelatin solutions and was first observed by Garrett (1903) The case of gelatin is complicated by the fact that this protein exists in two forms (Smith 1919) the first gelatin A stable above 35 with a value for $[\alpha]_D$ the specific optical rotation of — 313 and the second gelatin B stable only below 15 with a value for $[\alpha]_D$ of — 141 This variation of optical rotatory power with temperature occurs among proteins only in gelatin (Trunkel 1910) It only occurs between the temperatures of 35 and 15 above or below this range the value for $[\alpha]_D$ does not vary with temperature and Smith suggests that above 35 only gelatin A the sol form is present while below 15 gelatin B the gel form occurs Between these temperatures both forms will be present in a ratio depending on the temperature and age of the solution

In making a solution of gelatin by heating it in water the gelatin will dissolve entirely as the A form and viscosity measurements made immediately on cooling will be quantities determined for solutions containing a large amount of gelatin A and only small amounts of gelatin B The viscosity of solutions of gelatin A is constant at the iso electric point and decreases at all other reactions (Fig 28) After standing at temperatures below 35 however the proportions will be altered With the development of gelatin B in the solution the viscosity rises especially at the iso electric point (Fig 29) It does not necessarily follow from this that gelatin B at the iso electric point is more heavily hydrated and occupies a greater volume in the solution than either gelatin B salts or than gelatin A The viscosity formulæ of Einstein and Hatschek were deduced from experiments in the viscosity of oil water emulsions and show the influence of the relative volume of the dispersed phase in a liquid liquid system but

the viscosity of an emulsion can be altered without altering the phase ratio The addition of almost any solid substance which is adsorbed at the interface raises the viscosity of the system without altering the relative volume of the dispersed

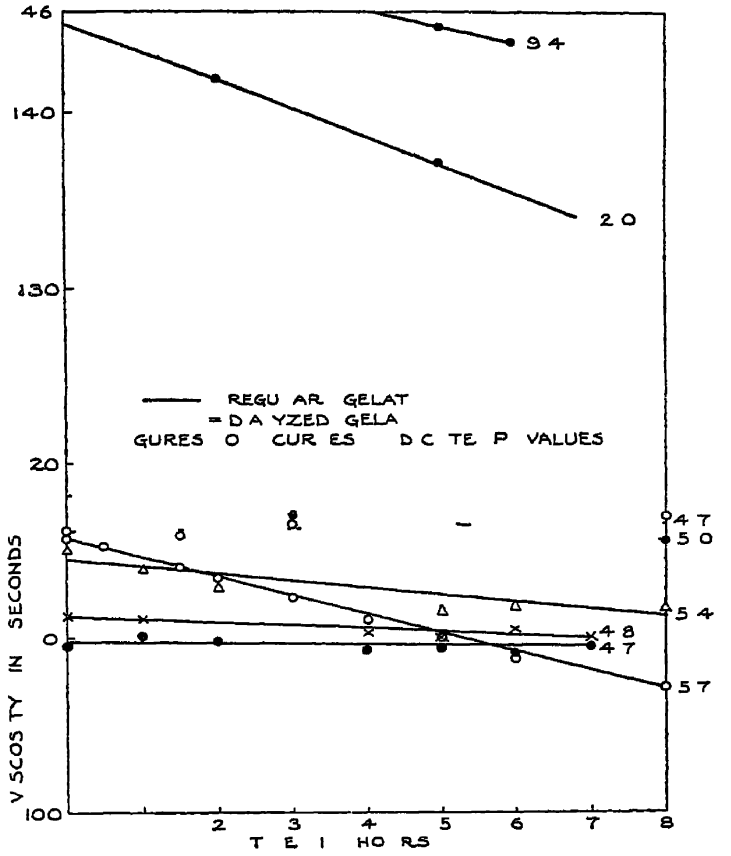

FIG 28 —Influence of time and *p*H on viscosity of solutions of gelatin (From Bogne *Journal of the American Chemical Society* 1902)

phase—lamp black in oil and water is a well known example and iodine in ether and water has the same effect of stiffening the emulsion The increase of viscosity of gelatin solutions with time which ultimately leads to gelation is probably due to the separation of finely dispersed precipitate of in soluble gelatin B throughout the system The precipitate

is continuous but open in structure (Jordan Lloyd 1920/2) and has been called by Bogue (1922) a system of catenary threads Bogue s measurements of the viscosity of gelatin solutions made by means of a rotating cylinder show that

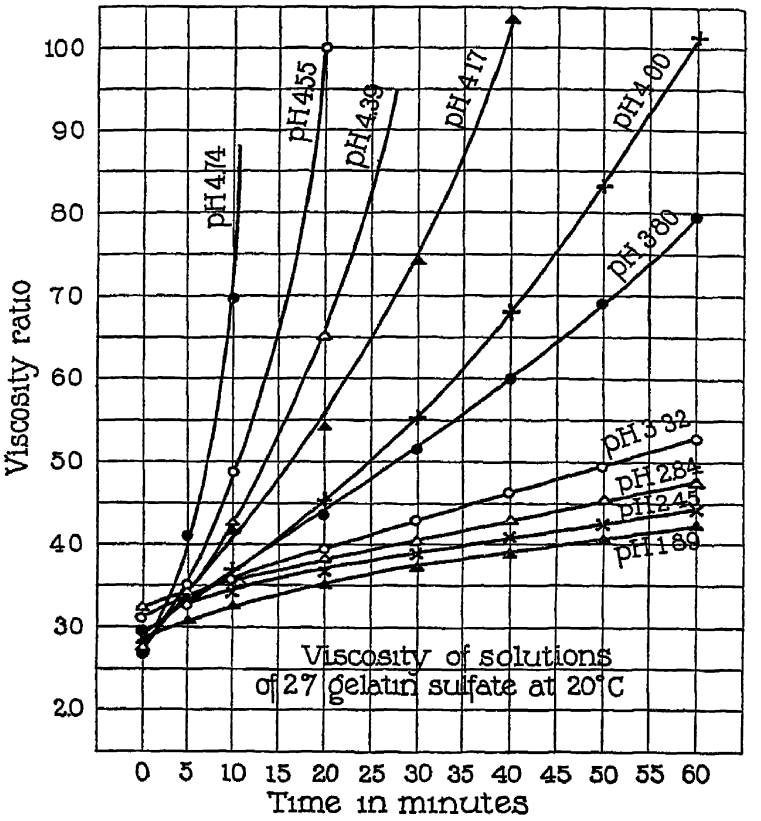

FIG 29 —Influence of time on viscosity of solutions of gelatin at different values of *p*H Temperature at 20
(From Loeb *Journal of General Physiology* 1920/1)

at temperatures above 35 they behave like typical viscous liquids while below that temperature they change their character to that of plastic solids

The Behaviour of Proteins on Drying

The increase in the tendency of proteins to form molecular aggregates as the solution becomes more concentrated is

clearly illustrated if a solution of a protein is allowed to evaporate to dryness The solubility of pure proteins is usually low (Jordan Lloyd 1920 Bradford 1918 1920 Cohn 1922) nevertheless they do not usually separate from a watery solution as a result of concentration (see however Bradford 1921)

If a solution of a crystalloid is evaporated to super saturation the dissolved substance normally starts to form crystals and a solid phase formed of crystals separates from the liquid On the condensation of a protein solution by evaporation it is only in a few limited cases that there is any separation of a second phase in the sense of Gibbs phase rule The dissolved protein clinging tenaciously to the last traces of water forms a continuous mass of aggregated protein particles and is finally obtained as a horny semi transparent mass of material This is the case not only with a protein like gelatin but equally with one like albumin and the tendency of the proteins to cohere is so strong that even protein precipitates which have been thrown down by strong salt solutions or alcohol will if they still contain water tend to cohere on drying Proteins can only be dried to fine powders by the use of alcohol or acetone both of which extract the water from the aggregate and simultaneously suppress the colloidal properties of the protein

The Distribution of the Water in the Blood and Tissues

The general transport agency of the body is the blood It brings raw materials to all the cells of the body removes waste products and regulates their content of water It circulates through a system of blood vessels many of which have diameters of capillary dimensions and walls sufficiently permeable to allow free diffusion of dissolved crystalloids It is of the greatest physiological importance that the circulation of the blood inside its own vessels should be maintained and to keep the necessary volume of fluid in these is a function

of the plasma proteins The osmotic pressure of the blood proteins has been measured by Starling The hydrogen ion concentration of the blood is a remarkably steady quantity in any one species In man it varies only between the limits of *p*H 7 3 and 7 4 and it therefore follows that the osmotic pressure of the plasma proteins is also a nearly constant quantity The importance of a sufficient concentration of soluble non diffusible matter in the blood becomes apparent under certain abnormal conditions After severe bleeding or in cases of physiological shock both of which appear to lead to a condition in which the volume of blood available is insufficient to fill the volume of the system of blood vessels there may be danger of the heart failing to act through an insufficiency of the blood supply coming in from the veins Under such conditions the action of the heart can be restored by transfusing into the blood stream a solution of sodium chloride isotonic (*i e* in osmotic balance) with the tissues If the reduction of blood volume has been severe the effect of such a transfusion is only temporary The salts from the transfusion fluid escape through the walls of the capillaries and with them also goes the water Bayliss (1916) showed that in such severe cases the blood volume could be maintained in the blood vessels by transferring a solution containing both salts and a dissolved colloid In order to avoid disturbing the water balance of the tissues and to maintain a normal rate of working for the heart the osmotic pressure and the viscosity of the colloidal solution was adjusted to the values of normal plasma Bayliss used a solution of gum acacia a colloidal carbohydrate as a transfusing fluid The success of his work is a proof of the importance of the osmotic pressure of the non diffusible colloids of the blood in maintaining the blood volume The fact that a colloidal carbohydrate can achieve this end illustrates again the thesis put forward in Chapter II that many of the animal proteins are not of importance on account of their protein nature but for their physical properties However having used

protein as a readily available material to supply the blood colloids the importance of a mechanism for maintaining the reaction of the blood at a constant level in order to avoid among other things variations in osmotic pressure and viscosity becomes obvious The osmotic balance between the blood and tissues will be referred to again in the next chapter

The Physical Chemistry of Bread Making

A domestic instance of the influence of the physical condition of proteins on the course of a process is found in the chemistry of bread making Wheat flour is a mixture of starch grains with two proteins—gliadin and glutenin In order to transform the flour into a suitable physical condition to use as a food it is kneaded into a dough with water permeated with gas bubbles to give the greatest possible surface for attack by the digestive enzymes and then baked a process which fixes the system and still further increases the digestibility of both the carbohydrates and proteins (see Chapter VI) During kneading starch grains and proteins absorb water and the glutenin develops in a high degree the coherent properties characteristic of nearly all proteins Wood (1907/8) has found that there are two factors involved in baking one affects the volume to which a dough can be distended by the gas bubbles produced in it the other determines whether a dough will stand up during baking or whether it will flatten in the oven Flours are graded commercially as strong if they make a firm dough that stands up well and keeps its gas weak if they make a flabby dough that sinks down and lets the gas escape The strength of a flour is now known to depend mainly on the physical state of its glutenin Wood and Hardy (1909) first showed that the coherence of the swollen glutin is affected by the concentration of the acids and salts with which it is in contact and Jessen Hanssen (1911) later proved that the hydrogen ion activity at which the dough showed greatest

strength coincided with the iso electric point of the glutin at *p*H 5 5 Most flours have a natural *p*H of 6 to 6 5 (Jordan Lloyd Clark and McCrae 1917) and part of the action of many acid salts such as alum or di hydrogen sodium phos phite formerly sold as flour improvers was undoubtedly due to their effect on the hydrion concentration of the dough Since the tendency of proteins to cohere or form aggregates is at a maximum at their iso electric point the coincidence of maximum baking strength with the iso electric point of the glutenin is not surprising

Sharp and Gortner (1923) state that the baking strength of any flour can be graded by the viscosity of a standard suspension in water a value which is also affected by the reaction of suspensions *i e* by the degree of hydration of the flour proteins

It might possibly be thought that physical condition was the only thing which differentiated one flour from another Woodman (1922) has recently shown however by means of racemisation curves that though the gliadin from all wheat flours seems to be the same protein the glutenins vary in chemical character between one wheat and another He suggests that Woods volume factor is due to this cause and the strength factor to physical conditions

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CHAPTER X

THE ABSORPTION AND LOSS OF WATER BY GELS AND TISSUES

Imbibition—Osmotic Swelling and Hydrogen Ion Concentration—Swelling and Valency—Gels in Saturated Water Vapour Syneresis—The Swelling of Tissues—Physiological Œdema—Chemical Basis of Swelling

Imbibition

It has been shown in the previous chapter that water can be drawn into or expelled from solutions of proteins by means of osmotic forces Other proteins not necessarily in solution but in the form of gels fibres powders or the tissues of plants or animals also have the power of absorbing water becoming swollen during the process It is interesting therefore to compare the conditions under which water is absorbed by proteins either as sols gels or in the dry state in order to see whether the same fundamental causes are at work in all cases

Dry proteins are hygroscopic Ordinary commercial gelatin for instance has a water content which varies with atmospheric humidity the amount of water driven off by heating it to 100 being usually about 15 per cent Gelatin immersed in water gradually swells or imbibes water exerting a considerable pressure in the process Posnjak (1913) found that the pressure was greatest at the beginning of imbibition and that it diminished as swelling proceeded until in the fully swollen gel the value fell to zero The relation between the pressure and the proportion of water present in the gel can be expressed by the equation

$$P = P_1 C^k$$

where P is the pressure C the concentration of gelatin in the

swollen gel and P_1 and k are the constants *i e* the pressure is greatest at the beginning of imbibition falls off rapidly during the early stages and more gradually afterwards Posnjak s experimental curve showing the relation between P and C for gelatin swelling in distilled water is given in Fig 30 Under these conditions $P_1 = 1\,2 \times 10^{-5}$ and $k = 3\,10$ from which it follows that when C = 850 (85 per

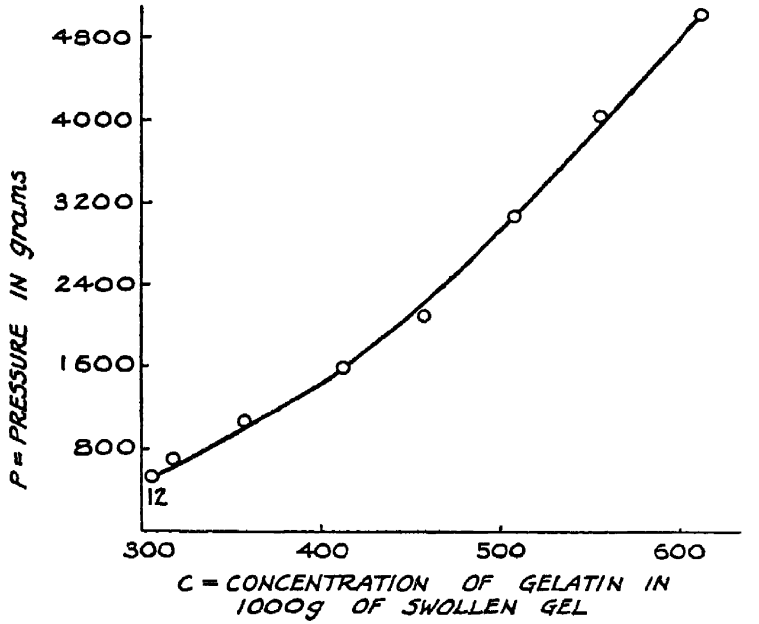

Fig 30 —The imbibition pres ure of gelatin swell ng n distilled water at 18 (From P snjak *Kolloidchemische Beihefte* 1912)

cent) the usual value for air dried gelatin P at the beginning of swelling equals 14 500 grams corresponding to a manome tric pressure of $1\,4 \times 10^5$ millimetres of water The most striking feature of this type of water absorption by proteins is the enormous value of the swelling pressure associated with a comparatively moderate increase in volume which according to Kaye and Jordan Lloyd (1925) is about seven fold It may be called *imbibition* Imbibition water is the water absorbed at or near the iso electric point by the

electrically neutral protein Imbibition is accompanied by an evolution of heat (Widemann and Ludekind 1885 Katz 1918) and a contraction in volume (Quincke 1870 Katz 1918) that is if the dry gelatin occupied a volume a the water absorbed a volume b and if c is the volume of the gel then $c < a + b$ Katz compared the imbibition of gelatin to the dilution of strong solutions of sulphuric acid or glycerine with water a process which is also accompanied by evolution of heat and contraction in volume of the two components

Imbibition water can only be driven out of gels by very powerful pressure Nevertheless it can mostly be readily removed by allowing the gel to evaporate in a dry atmosphere The evaporation and re imbibition of gelatin gels has been investigated by Brotmann (1921) Sheppard and Elliot (1922) and Hatschek (1924) Imbibition water is never completely removed except under conditions which usually also involve damage to the protein (probably anhydride formation) with loss of solubility The deformation of the drying gel is controlled by its geometrical form (Hatschek) and the volume and form of a dry gel after imbibition are also influenced by size form etc of the moist gels from which it was originally prepared (Hofmeister 1890 Pauli 1897 Brot mann 1921 Gortner and Hoffman 1922)

The imbibition of water by proteins at their iso electric points has been investigated only to a very small extent It is characteristic of all dry proteins and protein tissues Reinke (1879) measured the pressure volume curve for the imbibition of dried discs cut from the fronds of the seaweed *Laminaria* At 10 per cent moisture content he obtained a swelling pressure of 40 atmospheres *i e* a pressure equal to 5×10^5 mm of water a figure approximating very closely to that calculated from Posnjak s figures for dry gelatin

The association between protein and water of imbibition is a very close one and involves the loss of kinetic energy of many of the water molecules There is probably a condensa tion of water on internal surfaces in the gel and possibly even

combination at definite positions in the protein molecule There is also a penetration of water through the system which may be regarded as a solution of the water in the protein

Osmotic Swelling and Hydrogen Ion Concentration

Proteins which are in equilibrium with water at their iso electric points can be made to swell further by placing them in either acid or alkaline solutions This type of swelling is due to the development of osmotic pressure in the gel the surface of which acts as a permeable membrane (Procter 1914) Osmotic swelling contrasts with imbibi tion in the enormous volume increase that takes place accompanied by a comparatively small swelling pressure Imbibition gives a sevenfold increase of volume with leaf gelatin with a pressure of 1×10^5 mm of water Osmosis can give a further increase of 33 volumes in acid (pH 2 3) and 18 volumes in alkaline (pH 12 5) solutions (Kaye and Jordan Lloyd 1925) yet the maximum osmotic pressure developed by protein solutions does not exceed 150 mm of water (Smith 1921) or at most 500 (Loeb 1920–21)

In most of the experimental studies which have been made on the swelling of gels and tissues the total water absorbed has been the object study It was noticed very early that both acid and alkali increased the absorption and Ostwald (1905) Martin Fischer (1909) Pauli (1897) note that both the concentration and the nature of the acid affect the extent of the swelling The identity of the point of minimum swelling with the iso electric point of the gelatin was first noted by Chiari (1911) and subsequently confirmed by numerous workers The classical investigation of the effect of the *concentration* of the acid on the swelling was made by Procter (1914) and Procter and Wilson (1915) Procter followed the swelling of gelatin in different concentrations of hydrochloric acid by weighing the swollen gelatin and confirmed the results of previous workers that with increasing

concentration of acid swelling rose to a maximum and then diminished He also studied the distribution of both the hydrogen ion and the chlorine ion between the gel and the external solution Procter s work led him to believe that salt formation takes place between gelatin and the acid with the formation of ionisable gelatin salts and that the presence

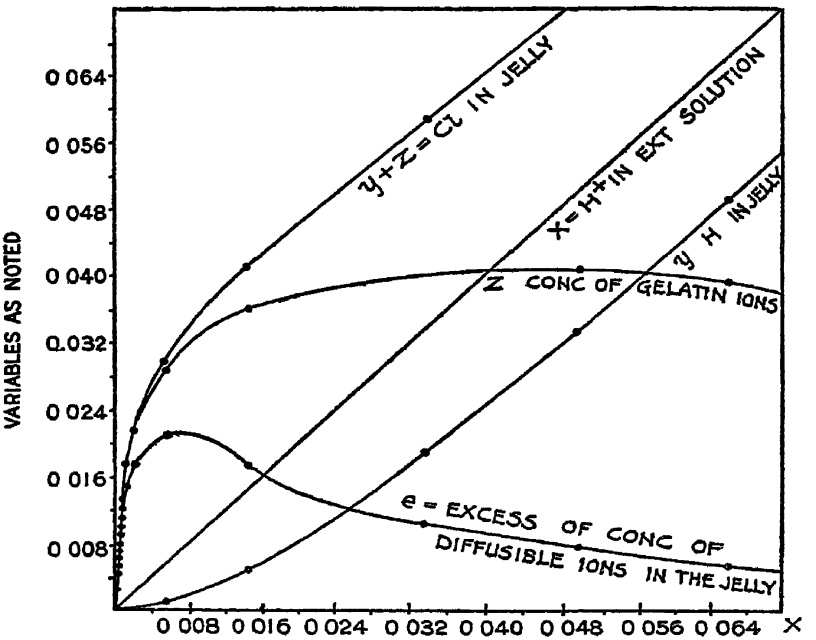

FIG 31 —Curves showing the relations between e z y and $y+$ and x the acid ty of the external solut on Abscissæ = x expressed as normality (From Procter and Wilson *Journal of the Chemical Society* 1916)

of a colloidal gelatin ion causes the establishment of a mem brane equilibrium at the gel water interface resulting in an excess of diffusible ions in the gel The unequal distribution of the ions leads to an excess of osmotic pressure in the gel which causes swelling Procter considered that the volume attained by the gel was limited by a factor due to the coherence of the gel itself but that it was directly proportional to e the excess of diffusible ions in the gel Procter and

Wilson give a diagram of the variations with x the external concentration of the hydrogen ion of y the internal concentration of the hydrogen ion $y + z$ the internal concentration of the chlorine ion and e This is reproduced in Figs 31 and 32 They show that

$$e = -2a + \sqrt{4x^2 + z^2}$$

and that therefore as a increases from 0 the value of e must first rise and after reaching a maximum fall again Their

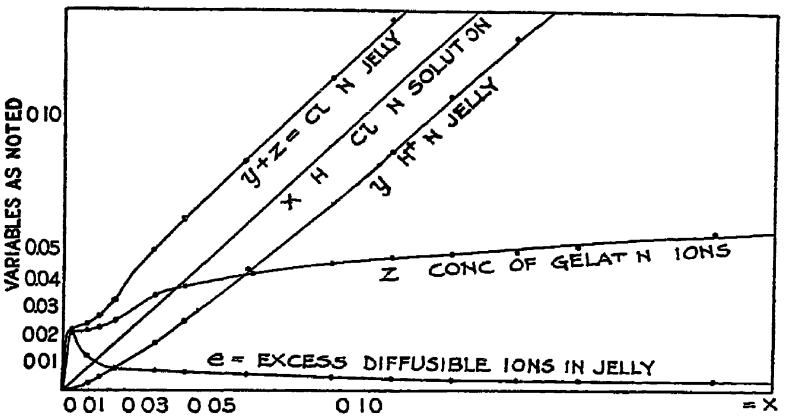

FIG 32 —Curves from Fig 31 shown over an extended scale (From Procter and Wilson *Journal of the Chemical Society* 1916)

theory explains the shape of the curve of swelling in acid solutions

The curve of gelatin swelling in solutions of hydrochloric acid and of sodium hydroxide is shown in Fig 33 taken from a paper by Jordan Lloyd (1920) It can be seen that the curve is in two branches one on either side of the iso electric point of the gelatin and that neither branch is a simple curve The acid curve rises sharply to a maximum at an external pH of 2 7 the alkaline curve rises slowly at first but later with increasing alkalinity it rises sharply to a maximum at pH 12 Both branches show secondary tendencies to rise again at reactions beyond pH 1 5 and 13 0 due to the first stages of solution in the acid or alkaline media It can be

seen at once that the acid swelling curve is in every way analogous to the curve of osmotic pressure shown on p 152 It was suggested by Jordan Lloyd that the volume of the gel is determined by a balance of two sets of forces the osmotic pressure due to the influence of the colloidal gelatin ion in the fluid phase of the gel and the elastic recoil located in the solid framework of iso electric gelatin Unless gelatin is present in both conditions the gel cannot be maintained When all the gelatin has been transformed into the salt form

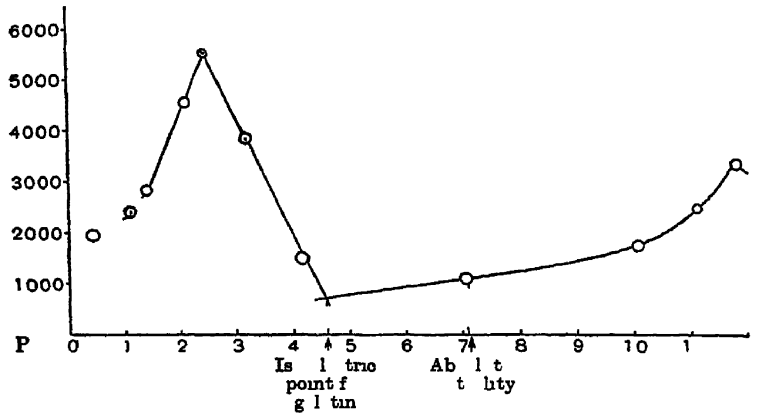

FIG 33 —Swelling of gelatin in solutions of hydrochloric acid (*p*H 0–6) or sodium hydroxide (*p*H 7–13) The ordinates represent the weight of 100 parts dry gelatin after forty eight hours at 18
(From Jordan Lloyd *Biochemical Journal* 1920)

the whole system dissolves (Jordan Lloyd 1920) when no salt form is present it shrinks to a minimum and if the protein is pure it separates out of solution (Jordan Lloyd 1920 Smith 1921 Field 1921)

The influence of the nature of the acid on the volume of the swollen gelatin has been studied by many workers Ostwald (1905) and Fischer (1909) both recorded that different acids evoke different degrees of swelling Loeb (1922) considered that swelling was affected not only by the hydrogen ion concentration but also in acid solutions by the valency of the anion or in alkaline solutions by that of

the cation Loeb considered that maximum swelling in all acid solutions was reached at an *internal* hydrogen ion activity of *p*H 3 1 and in alkaline solutions of *p*H 10 5 with minimum swelling at *p*H 4 8 The volume attained at maximum swelling was twice as great in the case of the monobasic acids nitric trichloraceic hydrochloric phosphoric oxalic as for the dibasic acid sulphuric (It must be remembered that Loeb brought forward evidence that phosphoric acid etc react towards the proteins like monobasic acids) Similarly the mono acid bases lithium sodium potassium and ammonium hydroxide cause twice as much swelling as the di acid bases calcium and barium hydroxide at the same internal *p*H value This generalisation of Loeb has been severely criticised Kuhn (1921) has examined the swelling of gelatin in the presence of sixty different organic acids and finds that although the total concentration of acid in the system at the point of maximum swelling varies inversely with the ionisation constant of the acid the actual volume at maximum swelling varies very greatly and has no connection with the basicity of the acid Ostwald Kuhn and Bohmer (1925) also found that the volume of maximum swelling was not related to the valency of the acid

The explanation of these apparent differences of opinion seems to be that Loeb s valency rule for swelling applies only to that fraction of the swelling that may be directly assigned to the osmotic pressure of internal ionisable protein salts But the total swelling of a protein gel is due not to one but a number of factors Not only has the salt formation between protein and acid to be taken into account but also the possible solvent action of the acid (or its anion) on the uncombined iso electric protein or in other words on its possible action on the coherence of the gel The solvent action of some neutral salts is considerable and there is no reason to suppose that a similar effect may not occur in acid solutions even though largely overshadowed by the

effect of the hydrogen ion The addition of neutral salts to an acid or alkaline solution has a very marked effect on swelling and will be dealt with in a succeeding chapter

The swelling of gelatin in acid and alkaline solutions has been dealt with above in detail since the type of behaviour found with gelatin is characteristic of all the proteins Among other proteins which have been studied in detail may be mentioned casein (Loeb 1920–21) collagen in the form of hide powder (Paessler and Appelius 1920 Porter 1921) fibrin (Fischer and Hooker 1918) and a number of protein tissues which will be dealt with separately in a later section

Gels in Saturated Water Vapour Syneresis

It has already been stated that the volume of a swollen gelatin gel is determined by the balance between the osmotic pressure of the diffusible ions in the liquid phase of the gel and the elastic forces due to the coherence of the iso electric gelatin forming the solid framework of the gel An interesting demonstration of this can be made by transferring gels swollen in either acid or alkaline solutions from the solution in which they have reached equilibrium to a saturated atmosphere of water vapour In the vapour phase the behaviour of the swollen gels may follow one of three courses (1) They may continue to absorb water from the saturated atmosphere and eventually go into solution (see Fig 34 Curves for N/2 and N/10 hydrochloric acid) in this case the gelatin may be regarded as present mainly in the salt form the iso electric solid framework having vanished and with it the elastic forces of recoil the gels behave like typical strong solutions of salts (2) They may maintain a steady weight (Curve for N/20 acid) (3) The gels may contract squeezing out water from their interior as fluid drops which drain away (Curves for N/100 000 — N/200 acid) This contraction which is very rapid becoming obvious after a few minutes only occurs after swelling in either acid or alkaline solutions In gels in equilibrium with N/200 hydrochloric acid a loss of

nearly a fifth of the water content may occur from this cause and in gels from N/200 sodium hydroxide about two thirds of the water is expressed This loss of volume from a system in which both gelatin salts and iso electric gelatin framework are present is due to the redistribution of the water and diffusible ions which follows according to the predictions of Donnan s membrane theory With the removal of the surrounding solution with which the gelatin gel had been in equilibrium the balance between the colloidal gelatin ions and the dif

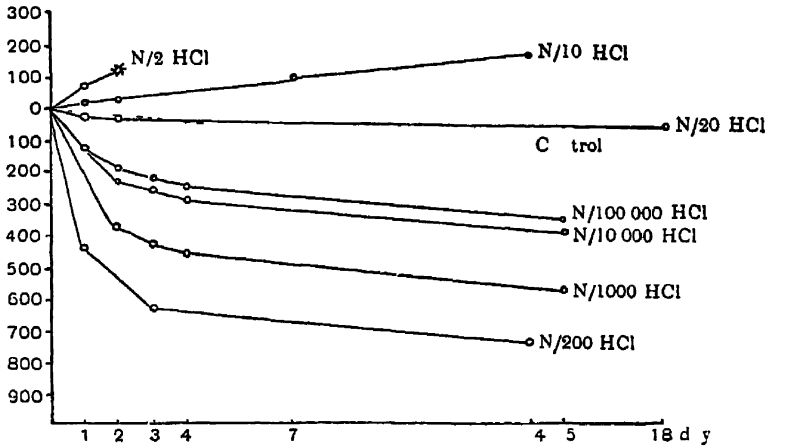

FIG 34 —The gain or loss in weight of swollen gels n equilibrium with hyd ochlor c ac d of vary ng concentration on being transferred to an atmosphere of satu ated water p u Ordinates = gain or l ss expressed as a percentage of the original dry w e ght of the gelatin (From Jordan Lloyd *Biochemical Journal* 19?0)

fusible ions of the same sign is disturbed A further quantity of the latter is therefore expelled from the jelly followed by their corresponding ions of opposite charge the osmotic forces in the gel are weakened and the elastic recoil of the gel leads to the squeezing out of the water

The spontaneous contraction of gels with extrusion of liquid is known as *synæresis* It is probably due in all cases to the osmotic forces leading to extension being less than the elastic forces leading to recoil Ordinary commercial gelatin only shows synæresis under the special circumstances

described above In gels of varying concentration allowed to set at nearly neutral reactions there is always a sufficient admixture of gelatin salts and other impurities for the internal osmotic forces to maintain the volume of the gel against the forces of recoil Highly purified gelatin however cannot maintain a stable gel form at reactions near to the iso electric point (Jordan Lloyd 1920 1921) The elastic framework of the gel is not counterbalanced by osmotic pressure at these reactions and is therefore free to contract and does in fact separate out from 1 per cent gels in the form of an opaque white clot in a clear watery fluid (Jordan Lloyd 1920 Field 1921 Smith 1921) The gelatin therefore retains only its water of imbibition In gels of higher concentration the transparent gel form is maintained (Smith 1921) since doubtless most of the water present is already in this form

The Swelling of Tissues

The swelling in water of dry tissues and of most tissues freshly excised from the body is very similar to that of gelatin depending on hydrogen ion activity and on the nature of the acid or alkali employed A tissue however differs from a gelatin gel in its structure It is composed of cells and each one of these acts as a separate unit The cells of a swollen tissue press on each other and so set up a mechanical pressure that acts against the osmotic swelling It is therefore not surprising to find that although the swelling of tissues is in many respects closely similar to the swelling of gelatin gels yet the volume attained under the same external conditions is always very much less in the tissue for every unit of dry weight The influence of hydrogen ion concentration on the swelling of the sterno cutaneous muscle of the frog has been investigated by Jordan Lloyd (1916) and the experimental curves obtained are reproduced in Fig 35 It can be seen how closely the curve resembles the curve of gelatin swelling shown in Fig 33

except in the magnitude of the ordinates Maximum swelling occurred at an external *p*H value of 2 2 in acid and 11 8 in alkaline solutions Minimum swelling was between *p*H 5 and 7 (Weber (1925) has recently stated that the iso electric points of the two muscle proteins are at *p*H 6 and 6 6 respec tively) Maximum swelling of frog s muscle gave a weight increase of 370 per cent on the moist weight or roughly 800 per cent on the dry weight of the muscle This is very

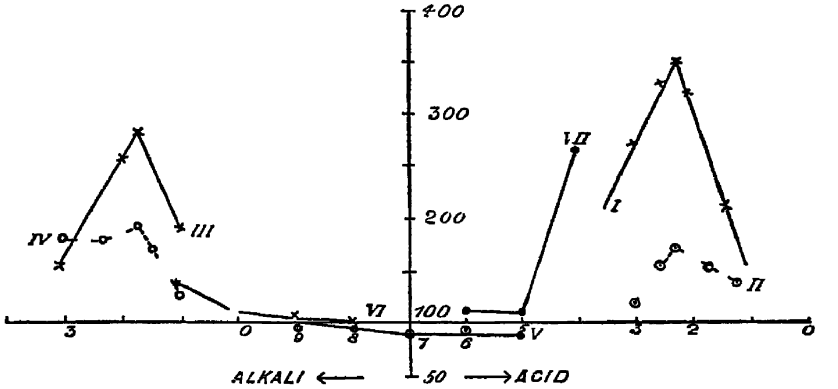

FIG 35 —Swell n f the sterno cutaneous muscle of the frog
I in hydrochl r c ac d
II in Ringer s s lution and hydrochlor c acid
III in sod um hydroxide
IV in R nger s solution and sodium hydroxide
V in M/15 phosphate buffer solution
VI in M/10 borate buffer olution
VII n M/5 acetate buffer solut on
Ord nates represent weight of 100 parts of moist freshly exc sed muscle
(From Jordan Lloyd *Proceedings of the Royal Society B* 1916)

considerably less than the 6 000 per cent attained by leaf gelatin With shaved goat skin a tissue much more compact and closely woven than the thin sterno cutaneous muscle of the frog maximum swelling in acid solution only leads to a 500 per cent increase on the dry weight of the tissue The curve of swelling of goat skin (Kaye and Jordan Lloyd 1924) is shown in Fig 36 Here again maximum swelling occurs as *p*H 2 2 in acid 11 8 in alkaline solution (The apparent maximum at *p*H 13 is due to secondary

causes) The contrast between the magnitudes attained by gelatin gels swelling freely and by collagen fibres swelling under the restrictions imposed by the fibrous interwoven structure of the skin is brought out in Fig 37 This difference is not due to chemical causes and can largely be obliterated by grinding the skin to powder It follows therefore that all tissues swelling under the influence of the hydrogen or hydroxyl ion are in a state of turgor each cell is exerting a considerable pressure on its neighbours due to the fact that

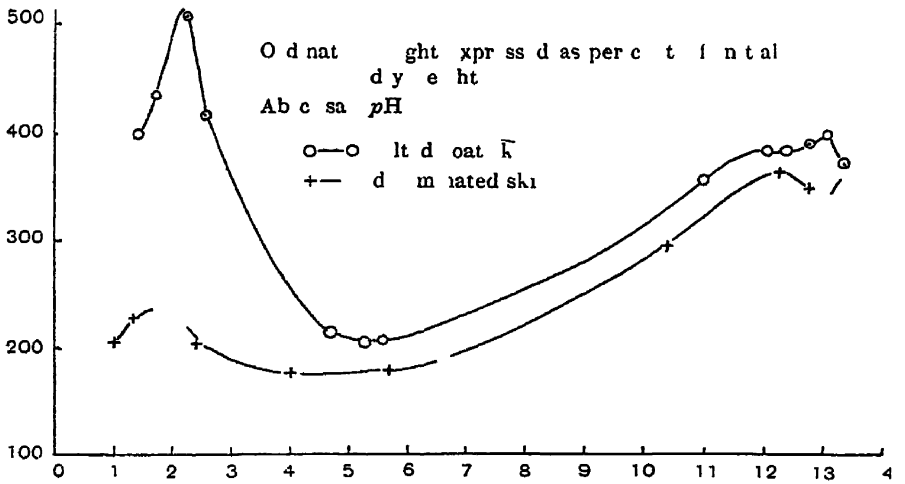

FIG 36 —Swelling of dried (shaved) goat skin in solut ons of hydrochloric ac d and of sod um hydroxide
(From Kaye and Jordan Lloyd *Biochemical Journal* 1924)

through the close approximation none can swell to its full volume

There is every reason to suppose that the influence of the hydrogen ion on the proteins of living tissue is the same as its influence on those of dead tissue since the swelling of freshly excised muscles which is accompanied by loss of contractility can be reversed in its early stages with a full return of contractile power The turgid swollen condition of a tissue can be produced not only in excised tissues by means of immersing in acid solution but also in tissues left *in situ*

in the living body Indeed Fischer (1910) considered that physiological œdema or tissue swelling was in all cases due to the colloidal swelling of the cell proteins under the action of

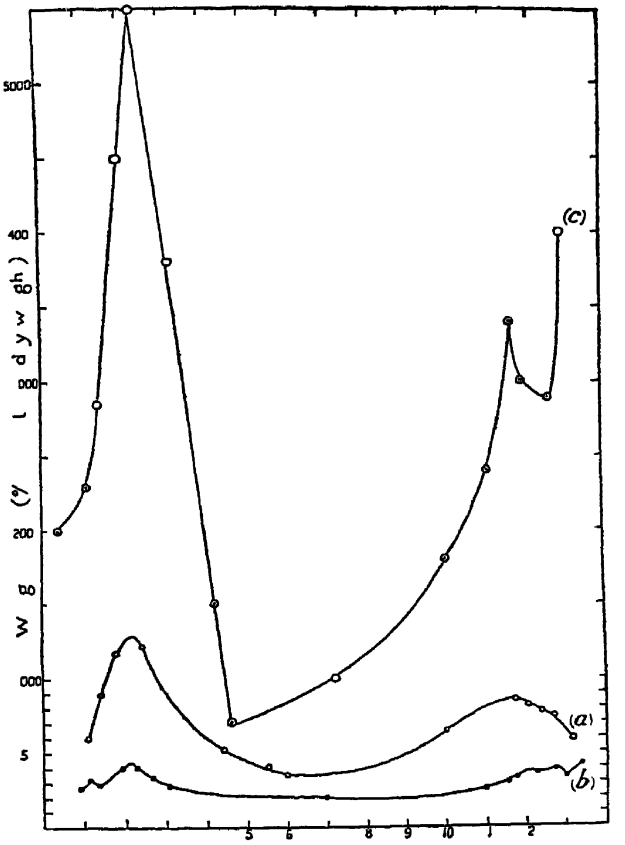

Fig 37 —Influence of hist logical structure on the comparative degree of swelling
Curve *a* fresh goat skin
b dried goat skin
c leaf gelatin
(From Kaye and Jordan Lloyd *Biochemical Journal* 1925)

an increased acidity inside the cell Normal healthy cells have a reaction which is not usually greatly removed from the point of absolute neutrality Needham and Needham (1925) give *p*H 7 6 as the value of the internal reaction of

Amœba proteus Vles and Coulon (1924) give the internal *p*H of the muscle fibres of white mice as about *p*H 6 0 Vles Achard and Prikelmaier (1923) that of the eggs of the sea urchin *Paracentrotus lividus* as 5 8 Under normal conditions the respiratory mechanism of the cells maintains the internal reaction at a fairly steady level Under other conditions however the acidity may rise Fletcher and Hopkins (1906–7) have shown that during activity as a result of stimulation lactic acid develops inside the muscle cell and that excised muscle if stimulated to fatigue swells more under equal conditions than an unstimulated one Barcroft and Kato (1915) and Back Cogan and Towers (1915) have shown that even with the muscles undisturbed from their position in the body and with the circulatory system intact a muscle absorbs water as the result of stimulation to fatigue Since swollen fibres increase in diameter but shorten in length a fatigued muscle contracted and turgid can cause actual physical discomfort or stiffness until normal conditions are restored It can be seen therefore that the cell contents may under conditions where the respiratory processes are out distanced by cell activity become turgid and swollen through the increase of the free acidity of the cell On the cessation of cell activity the lactic acid will be oxidised the *p*H return to normal and the absorbed water again expelled Fischer considered that all cases of physiological œdema could be explained on this theory and showed experimentally that by impeding the circulation to any tissue by ligaturing the artery the tissue increased in both weight and volume Fischer and Moore (1909) produced an artificial œdema of the kidney of the rabbit by this means Fischer (1910) also showed that putting a ligature round the hind leg of a frog induced œdema in the limb Becholdt in his book Kolloide in Biologie und Medezin extends the application of this theory to almost all known physiological manifestations It is certain however in the living tissue that conditions are more complicated than in a simple

colloidal system such as gelatin in aqueous solutions The lactic acid content of a muscle affects its water absorbing power but Foster and Moyle (1921) have also shown that living muscles made non irritable by prolonged exposure to cold have different osmotic properties from fresh muscles which cannot be accounted for by any change in the lactic acid content

Physiological Œdema

There is moreover in considering tissue swelling an important consequence of multicellular structure that must not be overlooked—a tissue as a whole may gain in weight and volume either as a result of water absorption by the individual cells or as a result of the accumulation of fluid in between the cells With skin it has been shown that the former condition which is due to the osmotic pressures of the contents of the fibrils leads to the tissue becoming swollen turgid and translucent while the latter leads to it becoming swollen white and flaccid (Kaye and Jordan Lloyd 1924) the absorbed water being not in but between the fibrils In the living animal body the blood which is the source of water supply to all the tissues is kept in the circulatory system by what is essentially a system of membranes and is like the cell contents under a definite head of pressure the arterial or blood pressure Substances which increase the permeability of either the cell membranes or the capillary walls will allow the contents to ooze away and to accumulate in the inter stices between cells or tissues The work of Dale and his co workers (1918 1919) has shown that an œdematous condi tion of this type can be produced by the action of histamine a substance which causes capillary distension with increased permeability of the capillary walls and is produced under certain conditions in the body The pathological accumu lation of fluid in the lymph spaces of the limbs and body cavity which is a clinical symptom for dropsy is more probably due to a constant leakage from the circulatory

system than to the development of an acid reaction in the cœlom as postulated by Bechhold Foder and Fisher (1923) consider at length the relation of œdema to membrane permeability and Schaede and Menschel (1922) also deal with the same question

Chemical Basis of Swelling

The swelling of gelatin and other protein systems which occur when these are transferred from neutral solutions to those of a more acid or alkaline reaction is due to the formation of soluble protein salts which ionise to form one colloidal and one diffusible ion and so set up an internal osmotic pressure

In acid solutions salt formation occurs at the free amino groups of the proteins If these groups are removed by nitrous acid the protein no longer swells to the same extent Skin for instance has its swelling in acid solutions greatly reduced by de amination though in alkaline solutions its behaviour remains identical with an untreated control The influence of previous de amination on the swelling of skin in hydrochloric acid and sodium hydroxide is shown by the curves in Fig 36 It is quite clear from the relative form of the two curves that de amination has not affected the swelling in alkaline solution but has very greatly reduced that in acid solution It seems therefore clear that the free amino groups of the protein take a leading part in the mechanism of acid swelling and by analogy it may be supposed that the free carboxylic groups are concerned with alkaline swelling

Certain proteins do not exhibit the phenomena of acid and alkaline swelling Apparently neither elastin nor reticulin swell either in acid or alkaline solutions It does not follow from this however that these proteins do not possess either free amino or free carboxylic groups The keratins (wool hair) do not swell in acid solutions yet undoubtedly possess free amino groups in their molecules These proteins swell

in alkaline solutions but it is not clear whether this swelling is an osmotic phenomenon similar to the alkaline swelling described for gelatin or whether it is due to the first stage of solution and hydrolysis by the alkali

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CHAPTER XI

THE INFLUENCE OF SALTS ON PROTEIN SOLUTIONS

Solubility of the Proteins in Neutral Salt Solutions—Peptisation— Salting Out —Influence of Salts on the Properties of Sols and Gels On the Position of the Iso electric Point On Solubility On Swelling On Osmotic Pressure Membrane Potentials and Viscosity—Physiological Effects of Salts Antagonistic Salts

Solubility of the Proteins in Neutral Salt Solutions

It has already been mentioned in the previous chapter that salts have a solvent action on proteins and exert a recognisable influence under nearly all conditions on proteins in solution Much of the experimental work on salt action has been made with the neutral salts of the alkalis and the alkaline earths

Neutral salts may be defined as those in which the total number of acidic valencies is equal to the total number of basic valencies The salts do not necessarily make neutral solutions in water and the expression is unsatisfactory for this reason In considering their influence on the properties of protein solutions it is therefore always necessary to keep in mind that changes apparently due to the salts may in reality be due to altered concentration of hydrogen ions The importance of this consideration has been dealt with at some length by Loeb (1922)

In dilute solutions (less than 2 or 3 Normal) neutral salts of the alkalis or alkaline earth metals increase the solubility of the proteins in water The salts are much less powerful solvents than are either acids or alkalis (200—500 times less)

and Hardy (1905) in his paper on the globulins showed that the mechanism of the solution is entirely different globulin dissolved by the action of salts being electrically neutral Hardy showed further that the amount of salt necessary to bring globulin to a transparent solution depended on the amount of globulin present and that over a range of globulin concentration varying from 0 19 to 0 95 per cent the number of equivalents of salt needed to dissolve unit weight of globulin was constant Though nowhere specifically stated it appears from Hardy s experimental figures that 1 gram molecule of sodium chloride can dissolve about 100 grams of globulin Cohn (1924) has recently shown that the rate at which solubility is raised by increasing quantities of salt is a characteristic for each individual protein He gives

$$S/S_0 = \alpha u^{\beta}$$

where S_0 is the solubility of the protein in water S the solubility in a salt solution the ionic strength of which is u and α and β are constants The solubility of a protein in a salt solution at or near its iso electric point varies not only with the concentration but also with the nature of the salt Hardy (1905) gives a list of salts examined by him in order of their increasing solvent power The figures express the weight of dry globulin held in solution by one gram equivalent of salt the value for sodium chloride being taken as unity —

KCl NaCl	1
KBr	1 4
NaBr	1 5
KNO_3 $NaNO_3$ NH_4NO_3	1 5
$Ca(NO_3)_2$ $Mg(NO_3)_2$	1 8
$BaCl_2$ $CaCl_2$ $MgCl_2$	1 8
K_2SO_4 $Na_2SO_4(NH_4)$ SO_4	2 0
$MgSO_4$	1 9
Potassium oxalate	2 1
Magnesium succinate	2 4
Sodium citrate	3 3

In the last three figures it is possible that changes in hydrogen ion activity may be influencing the results Hardy states that the solvent action of the salts when taken in pairs is equal to the sum of the action of each separately

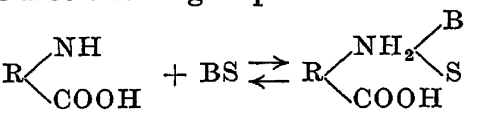
Peptisation

The influence of neutral salts on the solubility of proteins is responsible for a phenomenon known as peptisation This is the liquefaction of a protein gels or the dispersion of flocculent precipitates which takes place on the addition of salts The dispersal of the protein is a purely physical phenomenon is not accompanied by a chemical degra dation of the protein and is reversible on removal of the salts A worse term than peptisation with its suggestion of peptic digestion it would have been hard to find The peptic action of different salts under the same same conditions is different Posnjak (1912) noticed that he was unable to measure the swelling pressure of gelatin in 0 1 *N* potassium nitrate chloride or sulphate on account of the solubility of the protein under these conditions and that in 2 N potassium cyanide solubility was complete

Briggs and Hieber (1920) also record different degrees of peptisation by different salts With a 5 per cent solution of gelatin containing 33 per cent of salt (saturated in the case of ammonium chloride and potassium nitrate) they find that with potassium chloride there is rapid setting of the solution on cooling with ammonium or magnesium chloride delayed setting with zinc chloride potassium or ammonium nitrate or potassium thiocyanate no setting If the salts are removed by dialysis the sols set to gels If the salts are restored to the system it again becomes liquid

The chemical or physico chemical mechanism by which salts in neutral solution increase the solubility of proteins in water has not yet been satisfactorily explained Hardy (1905) postulated the formation of compounds containing

protein and salt chemically combined by linkage at the nitrogen of the free amino group —



Adolf (1923) considers that experiments on the conductivity of globulins in salt solutions support this theory and states that in N/10 solution four molecular equivalents of alkali chloride bind one molecule of globulin

On this theory a protein could combine with an acid or a salt but not with both Definite crystalline compounds are known between amino acids and neutral salts but convincing direct evidence of combination between proteins and neutral salts is still lacking

"Salting Out"

In concentrated solutions (3 to 5 *N*) the neutral salts act as protein precipitants This precipitation is not an electrical phenomenon but is apparently due to the reduction of the active mass of water molcules in the solution through the adsorption of the water molecules by the ions of the salt At a high concentration of salt this reduction of the available water is so extensive that the protein present is forced out of solution The concentration of salt necessary to initiate the salting out of a protein depends on the nature of the salt on the nature and concentration of the protein present and on the hydrogen ion concentration of the solution Chick and Martin (1913) have shown that in the salting out of crystallised egg albumin by ammonium sulphate all these factors come into p'ay and that both water and salt are carried down in the precipitated protein They show that maximum precipitation occurs at pH >5 in an experimental series covering a range from pH 4 to pH 9 Homer (1917) finds that in the precipitation of the euglobulin of serum by one third saturation with ammonium sulphate precipitation

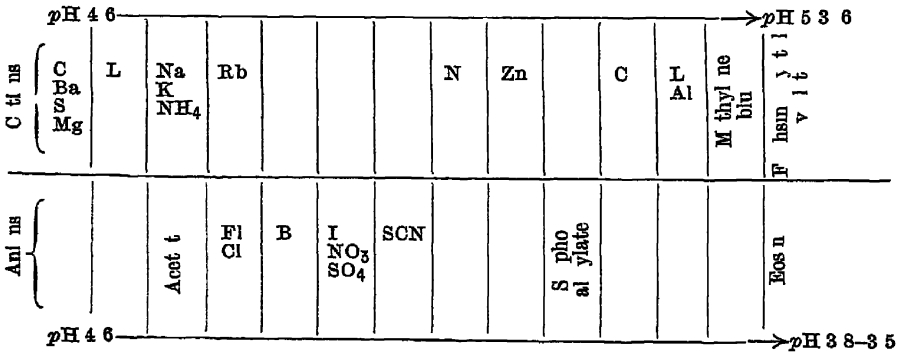
is least from *p*H 7 4 to 5 3 and increases in solutions either more acid or more alkaline The globulins which as a class are insoluble in water are freely dissolved by dilute solutions (up to about 2 N) of ammonium sulphate and completely precipitated by half saturation (about 3 N) of their solutions with this salt They are also freely soluble in 10 per cent (1 7 N) sodium chloride and in nearly all cases completely precipitated in saturated solution (33 per cent = 5 5 N) Bechhold gives 14 per cent (2 3 N) as the concentration of sodium chloride having the maximum solubility for globulins after which in more concentrated solutions the solubility is decreased Gelatin which is precipitated from solution by half saturation with ammonium sulphate is not salted out by saturation with sodium chloride Chick (1914) has suggested that the concentration of a salt necessary to salt out a protein from solution is inversely proportional to the volume of water that is associated with the dissolved protein

Influence of Salts on the Properties of Protein Sols and Gels

(1) On the Position of the Iso Electric Point —Hardy showed that in acid or alkaline solutions proteins were dissolved as electrically charged ions The point of minimum solubility he identified with the point of electric neutrality of the protein particles and called the iso electric point In his investigations on the globulins (1905) he showed that the position of minimum solubility (or maximum precipitation) is influenced by the salt content and that the effect of increasing salt (KNO_3) concentration is to shift the point of maximal precipitation over to the acid side Salts with bivalent anions caused the greatest shift and those with bivalent cations the least He showed moreover that the salt produced by bringing acid or alkaline solutions of globulin to the point of maximum precipitation by means of alkali or acid respectively also shifts the point on to the acid side from which it follows that if the method of minimum

solubility be used to determine the *p*H value of the iso electric point of a protein the result obtained will be influenced by the initial state of the system under considera tion Hardy s original observ ation seems to have been lost sight of for many years and many determinations of minimum solubility or minimum swelling made in the presence of buffer salts have been used to find values of the iso electric point Wilson and Kern (1922) even deduced the existence of two iso electric points for gelatin at *p*H 4 7 and 7 7 from their discovery of two points of minimum swelling in solutions of buffer salts though Atkin and Douglas (1924) later suggested that the appearance of the second point was due to the influence of the salts present

Michaelis and von Szeut Gyorgyi (1920) made a compre hensive study of the influence of salts on the flocculation of casein by acid or alkali The iso electric point of casein which in the absence of salts (or with only sodium acetate present) coincides with the position of maximum precipita tion is at *p*H 4 62 In the presence of salts the point of maximum precipitation may be anywhere between *p*H 3 5 and *p*H 6 Anions tend to move the point of maximum precipitation on to the acid side cations on to the alkaline side The following order was found for the influence of the salts —



The salts act according to the sum of the influence of their anions and their cations Sodium potassium or ammonium

acetate or rubidium fluoride or chloride give no shift Potas sium chloride at 0 1 *M* concentration shifts the zone from 4 4 to 4 7 to 3 8 to 4 4 the effect being due to the chlorine ion cupric chloride at 0 01 *M* shifts the zone to 5 3 to 5 6 *p*H the effect of the copper ion being predominating

It is important therefore that conditions should be very carefully controlled if the method of maximum flocculation or maximum precipitation is used to fix the value of the iso electric point It is interesting that Pauli and Modern (1925) using the method of alcohol precipitation on proteins purified by electrodialysis obtain values for the iso electric points of gelatin ovalbumin and serum albumin which are nearer to the point of absolute neutrality than those previously obtained by other workers The determinations of the iso electric point by Michaelis and his co workers were made by a direct observation on the drift of the protein in an electric field The influence of the salt concentration on these values has not been examined

(2) **On Solubility** —It has already been mentioned that in dilute solutions salts act as solvents for iso electric proteins and that in concentrated solutions near the iso electric point they act as protein precipitants The nature of the added salt strongly influences the amount needed to initiate precipi tation Hofmeister (1888 1891) showed that to produce clouding in a solution of white hen s egg by precipitation with the salts of sodium the following concentrations were needed —

	Mol pe l t e
Citrate	0 56
Tartrate	0 78
Sulphate	0 80
Acetate	1 69
Chloride	3 62
Nitrate	5 42
Chlorate	5 52
Iodide	∞
Sulphocyanide	∞

The order of the effective influence of the neutral salts has become known as the Hofmeister series It has been found to occur with minor variations under many different conditions The early experiments on the series were unfortunately made before the era of the direct determination of the concentration of the hydrogen ion and in the order of effective influence no allowance was made for changes in this induced by the salts The interlocking influence of acidity and salt action is shown by Hardy s (1905) observation that the addition of acid to a solution of globulin in potassium or magnesium sulphate lowers the solubility of the globulin in the salt solutions Similarly Moeller (1921) found that gelatin in solution in saturated sodium chloride could be completely precipitated by the addition of acid On the other hand the addition of sodium hydroxide increased the solubility of globulin in salt solutions at all concentrations (Hardy 1905) It is important therefore in studying the influence of salts on the solubility of proteins to make certain that the salt itself is not causing a change in the hydrogen ion concentration of the solution through its own hydrolysis The precipitation of electrically neutral proteins from solution by the salts of the Hofmeister series is attributed to the power which these have of becoming hydrated and reducing the active mass of the free water molecules—so called *lyophilic* action With proteins in solution as electrically charged colloidations not only the lyophilic influence of the neutral salts but also other factors come into play and the influence of the salts on the solubility depends on the relative concentration of acid and salt or alkali and salt as the case may be When the salt is present in low concentration it is possible to detect that salts exert an electrostatic influence precipitation of the ionised protein being due to the salt ion carrying the opposite charge the power of the salt being the greater the higher the valency of the ion The electrostatic precipitation of the proteins is however not a very well defined process It can be detected

only over a very short range of concentration for acid alkali or salt and it is greatly obscured by the solvent action due to the salts themselves (Hardy 1905)

(3) **On Swelling**—It has previously been stated (Chapter X) that many apparently insoluble proteins swell in distilled water by a process designated as imbibition In acid or alkaline solutions they swell through the action of osmotic forces due to salt formation The influence of salts on these two types of swelling is very different In general it may be said that salts increase the swelling due to imbibition but decrease that due to osmosis

The experimental investigation of the influence of the salts of the alkali and alkaline earth metals was initiated by Hofmeister (1888 1891) who examined the swelling of commercial leaf gelatin in solutions of various salts and placed them in an order corresponding to their effect —

SO_4 tartrate citrate < acetate < Cl < Br NO_3 < I < SCN

Hofmeister used salt concentrations of M/4 or greater in order to emphasise the difference between the salts On increasing the concentration sufficiently sulphates tartrates and citrates induced a shrinking of the gel compared with its condition in water acetates maintained the balance and the remainder caused swelling

Not only was a series found for the effect of anions but also one for cations —

$$Li < Na < K \; NH_4$$

Neutral salts also have a very marked influence on the swelling due to acids or alkalis in general depressing the swelling In Fig 35 p 183 is shown the curve for the swelling of frog s muscle in various concentrations of hydrochloric acid and sodium hydroxide and the influence which the presence of salts has on the course of swelling The salts added (Curves II and III) were sodium potassium and calcium chlorides in the concentrations equal to their concentration in frog s blood It can be seen

from the figure that the repressing action of the salts shows itself most strongly over the peak of the acid swelling and next in effect over the peak of alkaline swelling In strongly acid solutions (pH$<$1 5) the repression of swelling is slight and in the strongly alkaline solutions (pH$<$13) the presence of the salt actually leads to an increase of swelling In Fig 38 is shown a similar curve for the swelling of goat skin in hydrochloric acid and sodium hydroxide

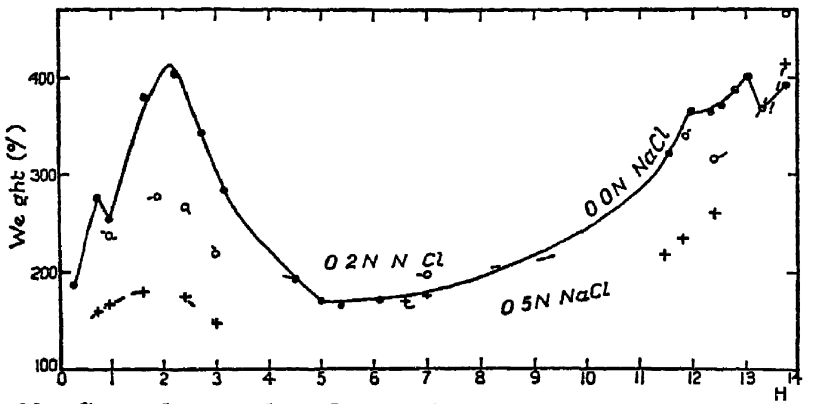


Fig 38 —Curve showing the influence of sodium chloride on the swelling of dry goat skin Note that the presence of the salt depresses the swelling due to the hydrogen and the hydroxyl ion Near the iso electric point and in strongly alkal ne solutions the presence of the salt increases swelling
(From Kaye and Jordan Lloyd *Proceedings of the Royal Society* 1924)

solutions in the absence of sodium chloride and at two con centrations of salt Here again it is shown very clearly that in acid solutions (pH$<$5) and in alkaline solutions over a limited range (pH 9 to 13) the salt represses swelling while near the neutral point and in strongly alkaline solutions it increases it

The influence of salts in the zones of maximum acid and alkaline influence has been the subject of a series of interesting experiments by Loeb who showed that in these two ranges the salts affected the equilibrium through one ion only—the anion in acid solutions and the cation in alkaline ones—and that the amount of the effect was a function of the valency of

the active ion His experimental curves showing the influence of salts on the swelling of gelatin are shown in Figs 39 and 40 It can be seen how the influence of the salt makes itself felt even at very high dilution (M/8000) At the two values of hydrogen ion concentration examined (*p*H 3

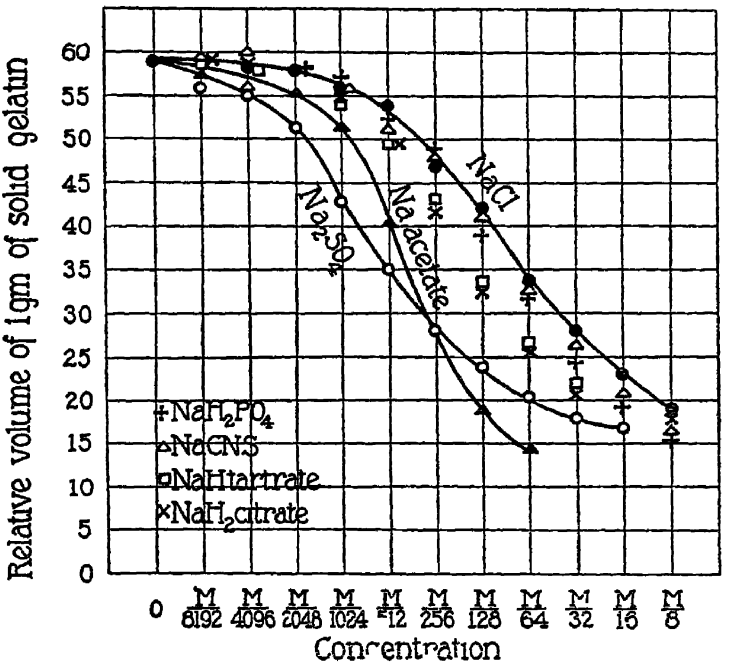


FIG 39 —Influence of salts on the volume of swollen gelatin at *p*H = 3 3 The curves show the effect of the concentration of the salt and of the valency of the anion
(From Loeb *Journal f General Physiology* 1921/2)

and 9) and at the dilutions of salts used the Hofmeister series of salt action is not found The repression of swelling is due to the disturbance of the membrane equilibrium between gel and surrounding fluid caused by the addition of a large number of diffusible ions The proportion of non diffusible to diffusible ions of the same sign is reduced by the addition of the salt with a consequent lessening of the Donnan effect at the gel surface and an equalisation of the distribution of both the positive and negative diffusible

ions and as a consequence of the water in the system The visible result of this is that the swollen gel loses water and shrinks The loss in volume is proportional to the amount of salt added and to the valency of its effective ion In an acid swollen gel the distribution of the diffusible ions between the

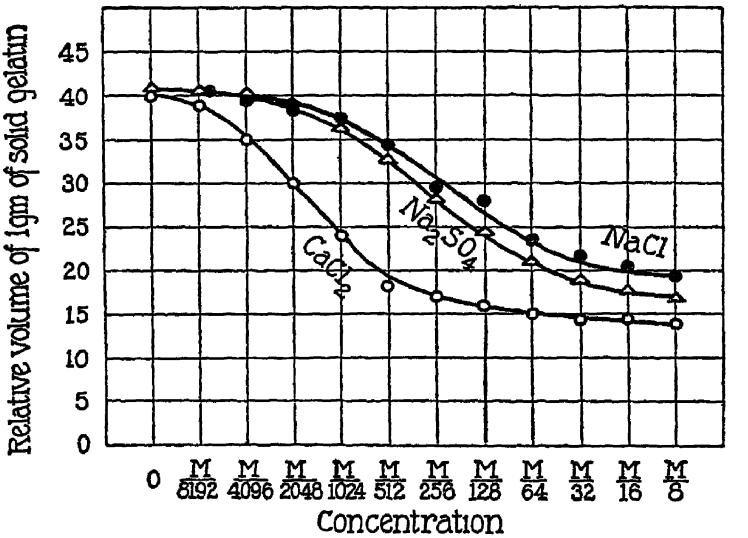


FIG 40 —Influence of salts on the volume of swollen gelatin at *p*H = 9 3 The curves show the effect of the concentration of the salt and of the valency of the cation

(From Loeb *Journal of General Physiology* 1921/2)

gel and the outer fluid hence both the osmotic pressure of the gel and its volume is influenced by the valency of the anion The influence of a salt added to such a system depends equally on the valency of its anion divalent anions having twice the depressing effect of monovalent at the same molecular (not equivalent) concentration and the valency of the cation of the added salt being without effect (see Fig 39)

In the alkali swollen gels the cation is the prepotent ion calcium having twice the effect of sodium independently of the anions which may be present in addition (Fig 40)

Loeb s experimental results confirm the statement made by

Hardy in 1905 that in acid and alkaline solutions and at low concentrations salts influence the state of proteins by means of their ionic charges

(4) **On Osmotic Pressure Membrane Potentials and Viscosity**—Loeb (1922) has shown that not only the swelling but also the other properties of the proteins influenced by hydrogen ion concentration are similarly affected by the presence of salts The osmotic pressure of protein solutions as recorded by means of a parchment membrane the viscosity of solutions of gelatin the electrical potential at the surface of a membrane gel or gelatin particle are all similarly affected by the presence of salts in proportion to the concentration and the valency of the added ions On all these properties the presence of the salt causes a depression of the property which had previously been augmented by the influence of the hydrogen ion The influence of salts on the viscosity at *p*H 3 is shown in Fig 41 *The influence of the salts is greatest where hydrogen ion influence is greatest and acts in the opposite sense* Loeb considers that the special characteristics of colloidal solutions (including protein solutions) are due entirely to the existence of a Donnan equilibrium not only at the boundary between the colloidal solution and any crystalloidal solution but also within the sol itself at the boundary of the particles of the dispersed colloid There is no doubt that this application of Donnan s membrane theory is justified for those proteins which exist in the sol state largely as molecular aggregates provided that it is applied only within justifiable limits that is to say only under conditions where the protein is present in the solution as an electrically charged ion of a protein salt In solutions where the protein is electrically neutral or in circumstances where it is present only as single molecules (not aggregates) membrane equilibria will no longer control the properties

Salts act on proteins (1) by their solvent action on the molecular aggregates of proteins (2) by the electrostatic effects of their ions Both these influences are probably

acting in all protein solutions but in most cases either one or the other predominates The swelling of gelatin in the absence of salts is a function of the hydrogen ion concentration having a minimum value at 4 7 to 5 0 and maxima at internal

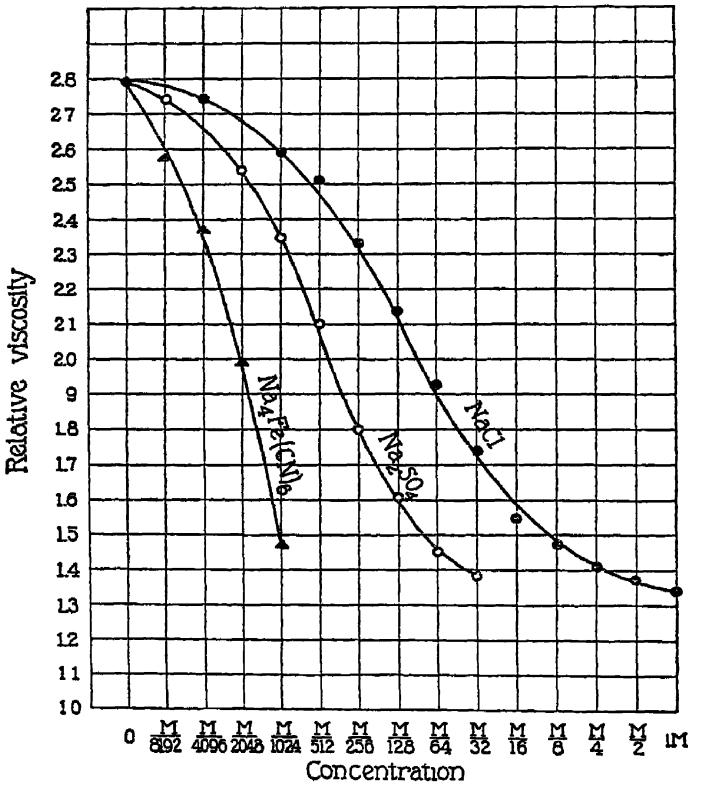


FIG 41 —Influence of salts on the viscosity of a 0 8 per cent solution of gelatin at *p*H = 3 The curves show the influence of the concentration of the salt and of the valency of the anion The relative depressing effect of chloride sulphate ferrocyanide = 1 4 16 Temperature = 24 (From Loeb *Journal of General Physiology* 1921)

*p*H values of 3 and 12 for strong acids and alkalis respectively On the addition of neutral salts swelling at *p*H 4 7 is increased the influence of the salt depending on its nature (Hofmeister series) swelling at *p*H 3 or 9 is repressed the influence of the salt depending on the valency of the oppositely charged ion (Donnan equilibrium) With weak acids as Ostwald Kuhn

and Bohme (1925) have shown these two effects may mask each other and the addition of salts may lead to increase of swelling at reactions other than the iso electric point In concentrated solutions of strong alkalis salts always increase the effects of the alkali The concentration in which the salt is present is also a factor in deciding whether electrostatic or lyophilic influences shall be more conspicuous in dilute solutions the former in concentrated solution the latter predominate but under all circumstances both influences are always present to a greater or lesser extent

Physiological Effects of Salts Antagonistic Salts

The experimental work recorded on the influence of the neutral salts on cells and tissues is so multitudinous in extent and variety that it is impossible to give any account of it within the limits of this book A very good review is given by Hober in Chapter IX of the fifth edition of his book Physikalischen Chemie der Zelle und der Gewebe The normal reaction of living cells lies very close to the iso electric point of the protein in some cases as in muscles a little on the acid side (Jordan Lloyd 1915) in others as in amœbæ (Needham and Needham 1925) on the alkaline side The proteins of the cells of plants and animals are therefore in the condition when they might be expected to show in their physiological behaviour towards salts the effect of the Hofmeister series Although in a great deal of the published work precautions have not been taken to stabilise the hydrogen ion activity and results due to this are entangled with results due to direct salt influence there is no doubt that in many cases salts have a direct action on cell proteins and the effect of different salts falls into the order of the Hofmeister series For instance the hæmolysis of the red corpuscles of mammalian blood was found by Hober (1908) to be in the following order of effect —

$$SO_4 < Cl < Br < NO_3 < SCN < I$$

Similarly Lillie (1910 1911) found that the pigmented eggs of the sea urchin *Arbacia* when placed in iso tonic salt solutions lost their pigment through an alteration in the permeability of the outer membrane the speed of diffusion outwards varying with the salt in the order

$$Cl < Br < NO_3 < SCN \quad I$$

Lillie found that the loss was more rapid in sodium than in potassium salts and in similar experiments with the pig mented larvæ of a marine worm *Arenicola* he gives (1909) the following order for the influence of the cations —

$$Si < Na < K < NH_4$$

Similarly in plant cells Trondle (1918) showed that salts affected permeability and turgor in the following order —

$$SO_4 < Cl < Br < NO_3 \quad I$$

and $Ca < Si < Br < Mg < Li < Na < K < Rb$

Antagonistic Salt Action —It will be noticed that in all the examples given the effect of the salt solution on the living cell is a deleterious one and that in spite of an osmotic balance the cell protoplasm is killed either by the solvent action of the salts on the cell proteins or by their coagulating effect either of which in neutral or nearly neutral solutions follows the Hofmeister series Any solution of a single salt even sodium chloride is toxic to living tissue and living cells are never in nature exposed to the action of a single salt The fluid contents of both plants and animals contain several salts in solution simultaneously and all of these are necessary for the proper physiological working of the living cells

The discovery of the antagonistic action of salts was first made by Ringer (1880–82 1882–83) working on the spon taneous contraction of the muscle of the frog s heart He showed that if an isolated heart were perfused with salt solu tion for the spontaneous beat to continue not only is it necessary to adjust the osmotic pressure of the perfusion fluid and in addition the reaction (faintly alkaline) but also the proportions of the salts present Ringer showed that in

an isotonic solution of sodium chloride the heart ceased to beat spontaneously but that the beat could be restored by the addition of calcium salts to the perfusion fluid To restore a normal rhythm however both calcium and potassium had to be present Calcium and potassium therefore antagonise the effects of sodium

The great value of Ringer s work to physiologists lay in the demonstration of the importance in all physiological experiments on excised tissues of keeping the tissues in contact with a normal salt balance Work on the composition of a physiological salt solution is due to Ringer and to Locke (1900 1901) The salt content varies for mammalian or for frog tissues For mammalian tissues the best composition is sodium chloride 0 9 per cent calcium chloride 0 024 potassium chloride 0 042 sodium bicarbonate 0 01–0 03 water 100 This composition leads to an atomic ratio of Na K Ca/100 4 1 The extension of the study of antago nistic salt action to other tissues is due largely to the work of Loeb (1911 etc) on marine invertebrate animals and to Osterhout (1906 1915) on marine plants Both these workers have shown that a proper salt balance is essential for maintaining the life of the organism

There is at present no satisfactory theory to account for the antagonistic action of salts on the behaviour of living protoplasm There is no doubt however that it is funda mentally due to the influence of the salts in the cell proteins There is sufficient evidence of antagonistic action in the domain of non living colloids to make this much at least certain Picton and Linder (1895) showed that in the precipitation by salts of colloidal arsenic sulphide while two mono valent cations or two divalent cations have an influence equal to the sum of each separately when mono valent and divalent cations are present together the precipitating action of the salts is less than the sum of the mono valent effect and the divalent effect or in other words that the ions act antagonistically Similarly Fenn (1916) has shown that in

the precipitation of gelatin by alcohol in the presence of chlorides the effect of sodium and potassium chlorides is additive but that the following salt pairs are antagonistic $NaCl + CaCl_2$ $NaCl + MgCl_2$ $CaCl_2 + MgCl_2$

It is an interesting fact that all the work on antagonistic salt action has been on the action of the cations As far as the writer is aware there is no evidence of antagonistic action among anions

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CHAPTER XII

THE THEORY OF PROTEIN SOLUTIONS

The Colloidal Nature of Protein Solutions—Electr (ı ɤ C)] Proteins—Electrically Neutral Proteins—The Properties of Protein Sols—The Properties of Protein Gels

The Colloidal Nature of Protein Solutions

A SOLUTION of a protein in water may vary in character from a clear transparent fluid (egg or serum albumin) to a cloudy dispersion (iso electric gelatin globulin) The length of the molecule of egg albumin has been determined by du Nouy (1924 1925) as $4\ 17 \times 10^{-7}$ cm that of serum albumin as $4\ 41 \times 10^{-7}$ and these figures may be taken as indicating the lowest limits of protein dimensions The diameter attained by particles of iso electric gelatin or serum globulin has not been so accurately determined but must be from $50\text{–}100 \times 10^{-7}$ cm since these particles are sufficiently large to scatter light rays The diameter of the water molecule or particle may be taken as $> 0\ 5 \times 10^{-7}$ cm The kinetic theory of solutions states that the kinetic energy of all the particles in a fluid is a function only of the temperature and is equal to $\frac{1}{2}mv^2$ m being the mass of the particle and v its velocity of translation In a solution of protein in water the greatest mass attained by the water particles may be taken as three times the molecular weight and is therefore equal to or less than 54 The mass of the protein particles varies from 10 000 to 100 000 or with molecular aggregates even more The velocity of translation of the

protein particles in a solution is therefore 50–200 (or more) times less than the velocity of the water particles A protein particle in solution therefore is a large slowly moving object strongly differentiated from the mass of water molecules surrounding it and the natural tendency of the protein may be assumed to be to separate out from watery solution under the action of gravity Even the single protein molecule is sufficiently large to show the beginnings of properties associated with matter in the mass *i e* the molecules possess surface and form a dispersed phase in the solution though they do not form a separate phase in the sense of Gibbs phase rule Hardy (1912) states that it is almost as erroneous to speak of colloidal solutions as multiphase systems without qualification as it would be to ignore their heterogeneity altogether Between protein and water however there is an interface which is the seat of interfacial and transfacial tensions and therefore possesses energy This interface in conformity with the third law of thermodynamics tends to diminish in area in order to decrease the surface energy This is accomplished by the cohesion of the protein molecules a process which if carried sufficiently far leads to their preparation from the solution

Proteins may however be kept in solution in water (1) by giving the particles an electrical charge thereby introducing a force which tends to extend the surface or scatter the molecules (2) by increasing the association of protein molecules with water molecules thus lessening both the interfacial tension and the difference in density between the two types of particle

Solution under the influence of an electric charge is a phenomenon characteristic of all forms of colloidal solutions whether of the suspensoid (irreversible lyophobe) type like the hydrosols of the metals or of the emulsoid (reversible lyophilic) type like the hydrosols of proteins starches etc Solution under the influence of an association of the colloid

with the solvent is characteristic of the latter class only and in the case of proteins may be brought about in several ways

Electrically Charged Proteins

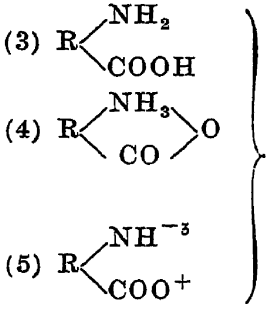
The physical aspect of protein solution can readily be correlated with the chemical properties of the proteins It has long been recognised that a substance will dissolve in a fluid when the forces of attraction between its own molecules are less than the forces between these and the molecules of the fluid In the case of an organic substance the chemical nature of its groups is the factor which determines solubility in any liquid and the two predominatingly active groups in the protein molecule are the amino and carboxylic groups Both these are strongly attracted by water probably because in solution they produce the same ions as the water itself The proteins as a class dissolve in water though the solubility of very pure preparations is low They are not generally soluble in pure organic solvents (see however Cooper and Nicholas 1925) Increasing either the hydrogen ion or the hydroxyl ion concentration of water (*i e* making the water either acid or alkaline) increases the solubility of the proteins by increasing their degree of ionisation or in other words by increasing their proportion of groups which are attracted into the water Proteins in the form of ionisable salts pass into solution therefore mainly as electrically charged particles

The protein may exist in solution either as the electrically charged forms

(1) $R\begin{cases}NH_3^+\\COOH\end{cases}$ } in acid solutions

(2) $R\begin{cases}NH_2\\COO^-\end{cases}$ } in alkaline solutions

or as the iso electric forms



in solutions near the neutral point

or

(1) Is the positive protein ion (2) the negative ion (5) the hermaphrodite or zwitter ion (3) and (4) are un ionised forms of the molecule of which (4) is probably the dominant type

In this amphoteric properties proteins resemble the amino acids The solution of proteins by acids or alkalis can therefore be regarded as due either to the formation of ionisable salts the solubility of which is greater than that of the protein base or protein acid taking part in their formation or else it can be regarded as due to the weakening of the interfacial tension between protein and water by means of the electrical charge given to the protein These are two aspects of the same question The electromotive potential developed at the interface of a protein particle with water may vary from 10 to 50 millivolts In solutions of proteins such as hæmoglobin or the albumins which apparently dissolve as single molecules the potential is entirely due to ionisation but with proteins such as gelatin casein or the globulins which appear to exist in solution as molecular aggregates there is also a membrane potential at the protein water interface and the actual value of the potential difference therefore although due primarily to ionisation will contain a factor due to the unequal distribution inside and outside the protein particle of the oppositely charged diffusible ion

The charged protein particles or protein ions can be precipitated from solution by conditions which reduce the

potential to less than 10 millivolts This reduction can be brought about for instance by dilute solutions of salts the active precipitating ion being the one carrying the opposite charge to the protein ion and having an effect which is proportional to its valency (Hardy 1900 1905) This precipitating action is with most proteins masked by the solvent action of the salts themselves It is very clearly marked in the case of solutions of denatured proteins the properties of

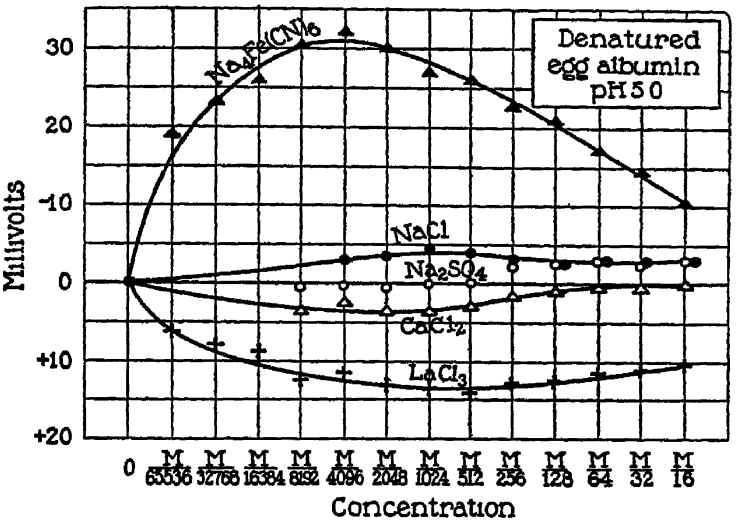


FIG 42 —Influence of salts on the surface potential of denatured egg albumin at its iso electric point
(From Loeb *Journal of General Physiology* 1922/3)

which are described later in Chapter XIII Tri and tetra valent ions at first precipitate proteins by discharging them and then redissolve them with change of sign The influence of the ions is so powerful that lanthanum or thorium ions can give a positive charge to proteins even on the alkaline side of their iso electric point (Chick and Martin 1912 Loeb 1922/3) The influence of salts on the surface potential of denatured albumin is shown in Figs 42 and 43 Fig 42 shows that at the iso electric point of the protein the quad rivalent negative ion of the ferrocyanide can give it a negative

potential of 30 millivolts while the trivalent lanthanum ion can give it a positive potential of 15 Fig 43 shows that at a *p*H of 5 8 where the particles would normally carry a negative charge the ferrocyanide ion is still able to raise the negative potential to 30 millivolts and the lanthanum ion can still confer on the particles a positive potential of 15 A point well illustrated in these two figures is that the electro

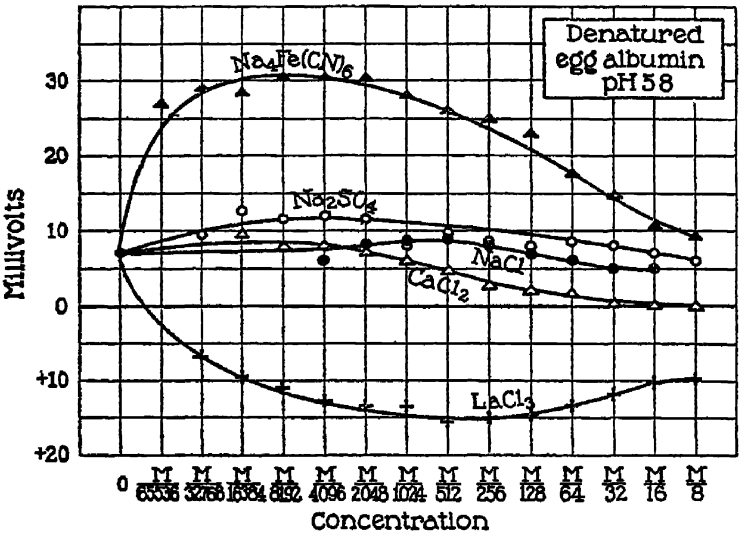


FIG 43 —Influence of salts on the surface potential of denatured e g albumin in solutions alkal ne to the iso electric po nt
(From Loeb *Jou nal of Gene al Phys l gy* 1920/1)

static influence of the salt reaches its maximum at a very low concentration

The addition of salts to solutions in which the protein is present as electrically charged aggregates leads to a re distribution of diffusible ions across the surface of the aggregates with a reduction of the membrane potential a diminution of osmotic pressure in the aggregates a diminu tion in their volume and a corresponding fall in the viscosity of the solutions In all these cases where the protein is held in solution by electrostatic forces the influence of salts on

the solution is an ionic influence and is a function only of the concentration and valency of the oppositely charged ion

Proteins however are not only held in solution by their electrical potential but their stability is also influenced by association with water (Hardy 1905 Pauli 1922 Loeb 1922) Even electrically charged proteins can in some cases be driven out of solution by substances which have the power of extracting this water from the particles such as alcohol acetone and strong solutions of certain salts the concentration of the lyophilic agent required varying with the hydrogen ion activity of the solution Loeb has designated as the alcohol number the number of cubic centimetres of 95 per cent alcohol required to precipitate completely the protein in 5 c c of a 1 per cent solution He has shown that the alcohol number has a minimum value at the iso electric point a condition obviously co related with the minimum degree of hydration that occurs at this point

Proteins held in solution by electrostatic forces behave like typical electrolytes They obey Ostwald s dilution law for binary electrolytes (Robertson 1907) The protein ions migrate in an electric field (Hardy 1905) and act therefore as conductors of electricity (Palmer Atchley and R F Loeb 1921) The rate of migration of a protein ion is a function of the hydrogen ion concentration of the solution Svedberg and Jette (1923) finding maximum velocity of migration for egg albumin solutions at *p*H 3 while the conductivity of all protein ions at infinite dilution is 50 reciprocal ohms (Pauli and Schon 1924 Adolph 1923)

Proteins differ from ordinary electrolytes however in having a large cumbersome ion which may arise from a single molecule or from several aggregated molecules The latter have been called pseudo ions (Hardy) or ionic micellæ (Loeb) They form a definite dispersed phase in the solution since they occlude water and diffusion of diffusible ions or molecules into them is controlled by the laws of membrane equilibrium

Electrically Neutral Proteins

A solution containing protein only in the form of ionised salts is however rather a theoretical than a practical possibility In very dilute solutions at special values of hydrogen ion activity it probably exists just as McBain (1925) has shown that soap solutions when sufficiently dilute exist as simple electrolytes free from colloidal micellæ In many solutions however protein is present as uncharged particles At the iso electric point the dissolved protein is all electrically neutral *i e* it is either uncharged or carries an equal number of positive and negative charges Solutions of this type are characterised by the tendency to aggregate formation leading to a high degree of turbidity and maximum ease of precipitation by alcohol or other lyophilic reagents

The formation of aggregates is favoured by the loss of the electric charge characteristic of protein in the ionised condition In one type of phraseology neutralisation of the electric charge is followed by aggregation and some times precipitation of the colloidal particles or in more chemical language the free ampholyte being less soluble than its salts can be precipitated from a solution by an adjustment of the hydrogen ion concentration It has been mentioned earlier that the protein water interface is the seat of special forces interfacial and transfacial The inter facial tension is a measure of the extent to which the protein tends to aggregate and separate from the water the trans facial is a measure of the extent to which the water tends to pull the protein into solution Evans and Bircumshaw (1924) have shown that the action of these two sets of forces is reciprocal and that the interfacial tension must be at a maximum and the transfacial tension at a minimum at the iso electric point On the theory of protein ionisation it is easy to see why this should be In the absence of any disturbing conditions the natural cohesiveness of all proteins can show itself Even at the iso electric point however substances other than acids and alkalis can influence the

forces of the protein water interface and the proteins can be made to pass into colloidal solution It is easy to see how this may happen for just as many substances can lower the interfacial tension between water and air so many can lower the interfacial tension between water and protein The solvent action of the neutral salts has already been mentioned This action is probably due to an influence

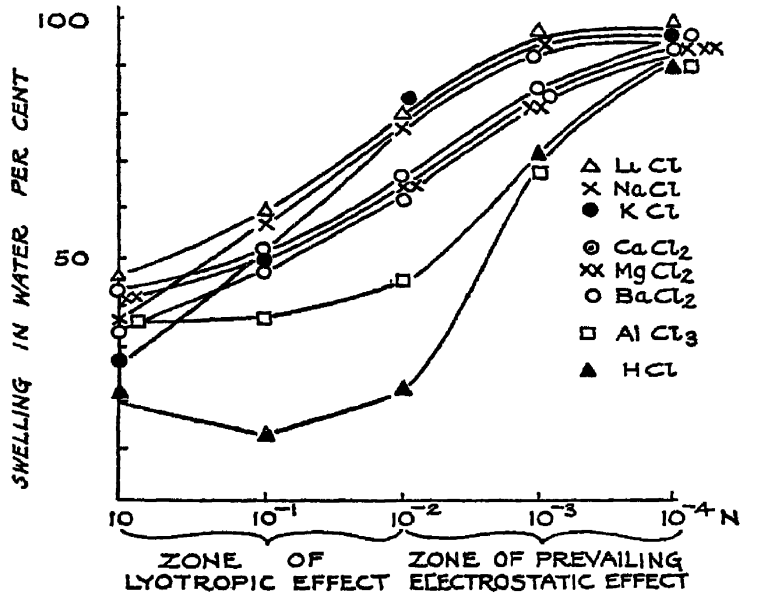


FIG 44 —Curves showing the effects of dilute and concentrated salt solutions on the swelling of air Comparative influence of the different anions

(From Dokan *Kolloide Zeitsch ft* 1924)

decreasing the interfacial tension and leading to an increased association of the protein with water and possibly to an increase in the formation of the zwitterions The influence of the salts on proteins in the iso electric condition is not due to their electrostatic properties but is in every instance a function of the nature of the salt and seems to be connected with the capacity of the salt ions to become hydrated *i e* to become associated with one or more layers

of water molecules adsorbed round the ion This lyophilic (or water attracting) capacity of the neutral salts is apparent in many of their influences on proteins but it is more clearly illustrated in Dokan s (1924) work on the swelling of agar a colloid free from the amphoteric properties of the proteins Agar is a colloidal carbohydrate and is weakly electro negative in aqueous solution Its maximum swelling occurs in distilled water and any addition of an electrolyte represses

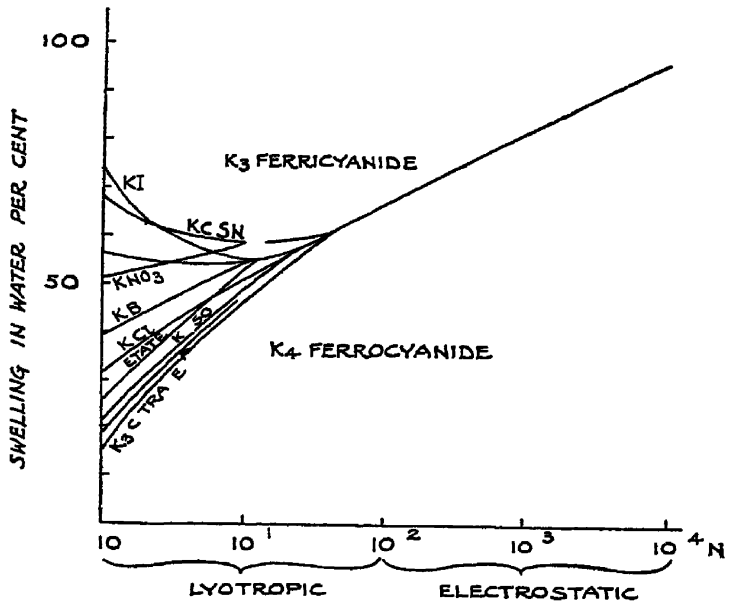


FIG 45 —Curves showing th effects of dilute and concentrated salt solu tions on the swelling of agar Comparative influence of the cations (From Dokan *Kolloide Zeitschrift* 1924)

the swelling Dokan s experimental curves are shown in Figs 44 and 45 which illustrate the influence of the cations and the anions respectively It can be seen that in very weak concentrations ($<10^{-2} \times$ N) the action of the salt is a function of the valency of the positive ion but that in higher concentrations ($>10^{-}$ N) the influence of the salt is independent of the valency of the ion and that in many cases the increase of the salt concentration beyond this

point is accompanied by a decreased repression of the swelling—*i e* after a certain concentration the presence of the salts causes the agar to swell This lyophilic action of neutral salts is even more graphically illustrated in Dokan s further work on Konyaku (1924) In the case of the proteins where the system is complicated by the amphoteric and electrostatic properties of the proteins the lyophilic effect of the salt is not shown to any extent until higher concentra tions have been reached but there is no doubt that the solvent action of dilute solutions of salts is due to their inducing an increased association of the protein and water Both sols and gels of highly purified gelatin are white and turbid (Jordan Lloyd 1921) and the addition of sodium chloride to the system causes a decrease in the turbidity which must be due either to the disaggregation of molecular complexes or to increased association of the protein with water with corre sponding decrease of the refractive index

Even proteins which do not dissolve in water have the property of absorbing water of imbibition and swelling It has already been pointed out that these swollen particles can be regarded as solutions of water in protein They constitute a dispersed phase into which any salts present are free to diffuse unchecked by any opposing influence due to colloidal ions The salts undoubtedly penetrate the colloidal micellæ—whether they combine with the protein is doubtful and is at present an unnecessary assumption They carry water with them according to their lyophilic properties and either by mere mechanical disruption or by reducing interfacial tensions they reduce the cohesion of the micellæ and so favour swelling and solution At higher concentrations (2N) the salt ions apparently adsorb water molecules to such an extent that there is a measurable reduction in the active mass of the water and the proteins are again driven out of solution This possibility is fully discussed by Michaelis in his book on The Effects of Ions on Colloidal Systems and it is a very striking fact that

not only in the solvation of proteins but also in their precipitation by strong salt solutions the same series though in reversed order is observed for the neutral salts namely —

SO_4 tartrate citrate $<$ acetate $<$ Cl $<$ Br $<$ NO_3 $<$ I $<$ SCN

for swelling and for precipitation —

SCN I $<$ NO_3 $<$ Cl $<$ acetate $<$ SO_4 $<$ citrate $<$ tartrate

Proteins precipitated from concentrated salt solutions always contain a fraction of the precipitating salt showing that there has been diffusion into the micellæ The lyophilic influence of neutral salts can be detected in solutions containing electrically charged protein particles but it is more conspicuous in solutions containing electrically neutral particles probably because of the smaller amount of water associated with the latter

Solutions of electrically neutral proteins have different properties from solutions of electrically charged proteins They neither show drift under an electric potential (Hardy 1905) nor do they contribute to the conductivity of the solution (Palmer Atchley and R F Loeb 1924) They have a high viscosity compared with ordinary electrolytes but a low one compared with solutions of ionised proteins They also have a greater surface activity Dissolved protein always lowers the surface tension of water but the effect is greatest at the iso electric point (Bottazzi 1909 Buglia 1908) and is therefore due more to the electrically neutral than to the electrically charged protein

The influence of surface forces in the equilibrium of protein solutions is well illustrated by the susceptibility of the latter to surface active reagents Soaps of the higher fatty acids for instance completely precipitate the proteins of serum or egg white (Matsumura 1923) Chloral hydrate completely precipitates the nucleoproteins of an aqueous extract of sheep s liver at a concentration of 3–3 5 per cent (Battelli and Stern 1913) Other anæsthetics (alcohols chloroform urethane etc) have a similar effect the concentration required in each case being inversely proportional to their power of lowering the surface tension of water

Ether chloroform and amyl acetate also cause the precipitation of serum proteins (Moore and Roaf 1903 1906) and it is difficult to see how their influence could be exerted except through a disturbance of the surface forces

The Properties of Protein Sols

In a solution of a protein therefore there may be electrically neutral protein particles and electrically charged protein ions The properties of the solution will depend on the relative proportions of these two forms In the presence of electrically neutral protein *i e* at the iso electric point

the lowering of the surface tension of water is at a maximum
the viscosity is at a minimum
turbidity is at a maximum
the precipitability by alcohol or acetone is at a maximum
osmotic pressure is at a minimum
conductivity is at a minimum

A consideration of these properties shows that proteins in solution at the iso electric point form comparatively small particles (low viscosity) only associated to a limited extent with water (high turbidity) and that this imbibition water although only with difficulty removed by pressure can fairly readily be extracted by means of another solvent— acetone alcohol or strong salt solutions Electrically charged proteins especially gelatin the globulins or casein which tend to form molecular aggregates in solution exist in solution as comparatively large particles (high viscosity) associated with very large quantities of water (low turbidity) part of which may be present as imbibition water part as drawn into the molecular aggregates by osmosis (Loeb) and possibly partly as water adsorbed on to the protein ions (Pauli 1922) The effect of an ionised salt on a solution of protein will in all cases depend on the ratio of ionised and un ionised protein The former will be influenced chiefly by the electrostatic properties of the ions notably by their

valency the latter chiefly by the lyophilic or water adsorbing properties These two effects may often work in opposite directions for instance the addition of sodium chloride to electrically neutral gelatin decreases the turbidity whereas its addition to ionised gelatin increases it (Jordan Lloyd 1922) Similarly the addition of salts causes increased imbibition or swelling at the neutral point but decreases it at other reactions With ionised protein the electrostatic influence of the salt is the predominating effect with un ionised protein the lyophilic influence is predominating but in no case will either influence be completely absent

The effect of non electrolytes on protein solutions will depend on their water absorbing power and surface activity

The Properties of Protein Gels

A very striking property of certain protein solutions is that they can become transformed into gels The gel condition the general features of which are familiar enough to anybody is only found with certain proteins Casein in alkaline solutions can form a gel similarly globulin in alkaline solutions can set to a clear stiff gel but the most familiar protein gel is the heat reversible gel of gelatin The mechanics of gel formation have been the subject of much discussion In the case of soaps the theory has been put forward that the setting of the sol to a gel is the reversal of a solution of soap in water to a solution of water in soap (Fischer 1924) This same idea is reflected in Bogue s (1922) theory of gel formation which assumes that the gelatin mole cules tend to adhere to form long chains that hydration of these takes place and that when a sufficiently high propor tion of water from the solution has passed into solution in the threads gel formation takes place On Bogue s theory rigidity of the gel is a limiting case of extreme viscosity due to the swollen gel particles having come to occupy the whole or nearly the whole volume of the gelatin water mixture

A somewhat different theory of gel formation due originally

to Hardy (1900) is that the formation of the gel is due to the separation of a solid framework throughout the solution This idea has been found from time to time in the literature under different forms Hardy originally considered that the gel framework was a symmetrical 3 dimensional net structure being actually a solid solution The writer (Jordan Lloyd 1920) impressed with the very low solubility of highly purified gelatin in water considered that the iso electric or neutral protein separated from the solution as a continuous framework and that the properties of any gel depended on the proportions of precipitated insoluble iso electric gelatin and soluble ionised gelatin present This theory of gel formation gives a model in which a solid framework is permeated by capillary spaces filled with fluid The geometrical form of the framework does not seem very important Hardy originally postulated a 3 dimensional net on evidence obtained from microscopical studies on the setting of solutions of gelatin in alcohol and water But in the case of soap gels it has been shown by Laing and McBain (1920) that the insoluble soap separates as long threads with quite irregular orientation Hatschek (1914) showed that gas bubbles generated in a gel have not a spherical but a lenticular form and that the angle at which these flattened bubbles lie relative either to each other or to the walls of the containing vessel is entirely without co ordination It seems more probable on the whole that the gel framework is an irregular tangle of threads He (1916 1917) has also shown on mathematical grounds that a gel cannot be a dispersion of one liquid through another a suggestion that was put forward on the evidence of the nature of the viscosity of protein sols—evidence which is not however incompatible with the solid liquid theory of gel structure (see Chapter IX) Callow s (1923) work on the formation of permanent tracks during ice crystallisation in super cooled gels provides interesting and novel evidence of the existence of solid structure in the gel

The most important property of a gel is its mechanical

rigidity combined with a remarkable lack of interference with the movements of the ordinary dissolved molecules of crystalloids For instance the diffusion of electrolytes through a gel takes place at a rate very little less than that through a column of water The diffusion of such substances through water is influenced by the viscosity the rate falling with increasing viscosity Yet in spite of this fact in highly viscous protein sols and in gels diffusion proceeds at a considerable speed Graham (1862) noticed that sodium chloride diffuses through gelatin at practically the same speed as for water and de Vries (1884) records the same fact for copper sulphate The question has recently been re investigated by Stiles and Adair (1921) They studied the diffusion of sodium chloride into gelatin gels containing silver nitrate The presence of the second salt acted as an indicator of the progress of diffusion Stiles and Adair give for the penetration of sodium chloride into a gelatin gel containing 0 01 *N* silver nitrate

Coefficient of diffusion in C G S units at 20 C —

4 per cent gelatin gel	$1\ 254 \times 10^{-5}$
8	$1\ 152 \times 10^{-5}$
16	$0\ 934 \times 10^{-5}$
Water (extrapolated value)	$1\ 41 \times 10^{-5}$

It can be seen that in spite of the enormous viscosity of the gelatin gel a viscosity too great even to be measured the rate of diffusion is reduced by less than 2 per cent for every gram of gelatin added to 100 c c of gel In gels less concentrated than 2 per cent the influence of the concentration of the gelatin is rather greater and Stiles and Adair suggest that in concentrations up to 2 per cent the protein is partly increasing the viscosity of a liquid phase but at greater concentrations it is only adding to a solid phase already present

The possibility of the capillary structure of gels which is one of the earliest theories of gelation and originally put forward by van Bemmelen (1898) to account for the properties

of silicic acid has further evidence thrown on to it by other diffusion experiments It was early recognised that crystalloids with their small molecules could penetrate through gels with a speed only slightly less than their speed in water but that colloids with their large molecules or molecular aggregates were quite unable to penetrate into a gel Ruhland (1912) examined the penetration of a large number of dyes into protein gels and grouped them according to their rate of penetration He found that the crystalloid dyes such as methylene blue penetrated the gel as rapidly as a column of water another group which he called semi colloid only penetrated very slowly and finally the colloidal dyes such as Bismarck brown and night blue were unable to penetrate into the gel at all The inference drawn is that the gel capillaries are too fine to allow the passage of colloidal particles

Further evidence of the internal condition of gels is obtained from certain other diffusion experiments Liesegang (1898 1906) observed that if potassium chromate were allowed to diffuse into a gelatin gel containing silver nitrate the resulting precipitate of silver chromate was not continuous through the gel but appeared as a series of rings These rings now called Liesegang rings are formed by certain salt pairs but not by all possible pairs of reacting salts A very large literature has grown up on the theory and practice of their mode of formation From the point of view of the capillary theory of gel structure one of the most interesting is the recent paper by Broderson (1924) showing that diffusion of two reacting salts into a narrow space of capillary dimension also leads to the appearance of banded precipitates Banded precipitates can be formed very slowly even in water (Zacharias 1924) though the gel or capillary state undoubtedly favours their formation

The theory of the mechanism of the formation of Liesegang rings belongs more to the study of the processes of diffusion than to that of the properties of proteins The appearance of the rings however has a considerable physiological

interest since a certain number of banded deposits are known in the plant and animal body Gall stones for instance which are accumulations of cholesterol (often with traces of pigment) formed in the gall bladder have a concentric structure due probably to the precipitation of the cholesterol in a colloidal medium The secondary rings visible in a section of a tree trunk between the primary annual rings may also possibly be of a similar nature The deposition of the calcium salts of bone may also be influenced by the colloidal nature of the matrix On the other hand the suggestion that the eyes on the wings of butterflies or the tail feathers of a peacock may be Liesegang rings is probably due to excess of zeal

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CHAPTER XIII

DENATURATION AND COAGULATION

Denaturation and Coagulation—Denaturation by Heat—Denaturation by Light—Denaturation by Strong Acids Alkalis and the Salts of the Heavy Metals—Denaturation by Mechanical Means—Denaturation by Alcohol and Acetone—The Action of Lecithin—Theories of Denaturation—Denatured Proteins in Physiology and Industry

Denaturation and Coagulation

It has been seen in the previous chapter that proteins can form in water solutions which vary in character from transparent molecular solutions similar in many ways to those of crystalloids to cloudy dispersions in which the evidence suggests that the protein molecules remain more or less aggregated In all cases there is some association between protein and water and by altering the concentration and nature of the electrolytes present the protein can be precipitated or dispersed either process being completely reversible Under certain conditions which will be given in detail below albumins and globulins can be *coagulated* or precipitated from solution by a process which is irreversible Coagulation is a physical condition and by suitable means coagula can be redispersed or dissolved The solution of coagulated protein however differs very definitely from that of the original material from which it came Coagulation is not in itself an irreversible process but in all cases is preceded by an *irreversible chemical change in the protein known as denaturation* (Hardy 1899)

Under the heading of denaturation can be included a number of reactions the common features of which are a

complete loss of solubility in water and in dilute salt solutions Denatured proteins however are readily dissolved in dilute acids or alkalis giving viscous colloidal solutions which react towards electrolytes as if they were of the suspensoid type rather than the emulsoid type characteristic of normal proteins The change involved in denaturation seems to be a structural alteration in the protein molecule which leads to a re arrangement of the linkages in the molecule but not an actual degradation This change is accompanied by a complete loss of the power of swelling by imbibition of water Denatured proteins are completely insoluble at their iso electric points even in the presence of salts They can only be dissolved in the ionised condition the solutions have the character of suspensoid colloids (Hardy 1899) and reprecipitation can be brought about from either acid or alkaline solutions by ions of the opposite charge in accordance with Hardy s valency law Proteins can be denatured by strong acids or alkalis by salts of the heavy metals by heat by light by mechanical agitation by pressure or adsorption on a surface and by the action of alcohol or acetone The inter relation between different types of denatured proteins has not yet been worked out

Denaturation by Heat

The type of denaturation which has been studied in greatest detail is that which occurs under the action of heat This is characteristic of the two groups albumins and globulins Under many conditions it is accompanied by the coagulation of the denatured protein The commonest example of this is of course the setting of the white of an egg which takes place on boiling Denaturation and coagula tion are both influenced by temperature time the reaction of the solution the presence of water and by the nature and concentration of the electrolytes present but the effect of these factors is different for each of the two processes

Denaturation is a process which cannot occur except in

the presence of water Chick and Martin (1910 1913) showed that crystals of egg albumin squeezed as dry as possible between filter paper could be heated for five hours in a current of dry air at 120 without losing their solubility Heated with steam at 120 the protein became totally insoluble in a few minutes Heated in aqueous solution under conditions arranged to ensure a constant hydrogen ion concentration it was found that the concentration of the

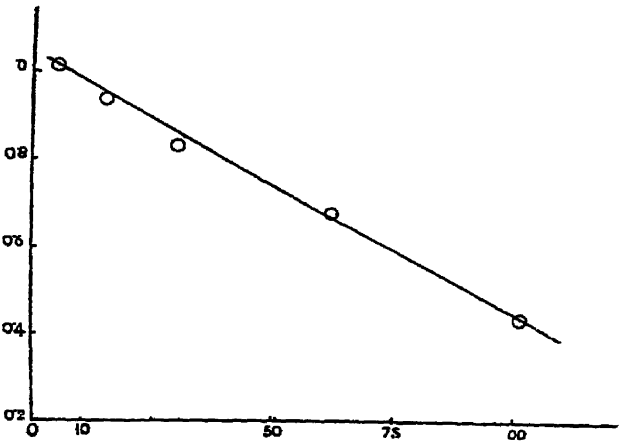

FIG 46 —Coagulation of egg albumin in presence of saturated boric acid at 51 1 pH = 3 1 Ordinates = log (concentration of residual albumin in mg per c c) Abscinæ = time in minutes
(From Cluck and Martin *Journal of Phys ology* 1912)

unchanged albumin in the solution was a logarithmic function of the time of heating *i e* the denaturation rate at any moment is proportional to the concentration of the un changed protein The experimental curve showing the progress of denaturation at 51 1 in saturated boric acid is reproduced in Fig 46 Chick and Martin (1911 1913) find that under these conditions the velocity constant of the change calculated from the equation

$$K = \frac{1}{t - t_0} (\log C_0 - \log C)$$

is equal to 0 00074 They conclude that they are measuring directly the reaction rate of denaturation since at the reaction chosen and in the presence of the small amount of ammonium sulphate carried over from the mother liquors during crystallisation the precipitation of the denatured albumin will be much more rapid than its rate of production

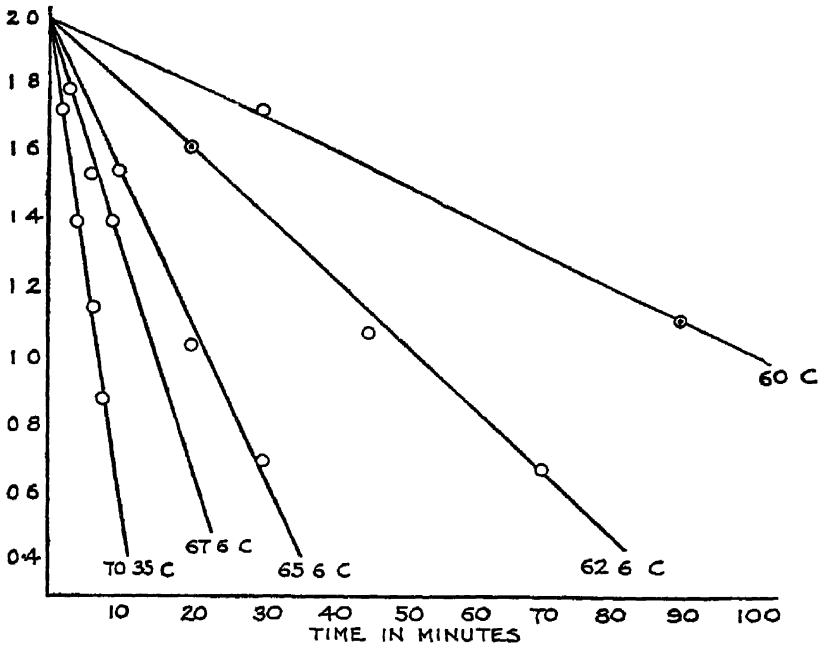

FIG 47 —Influenc of temperature on the rate of denatur tion f a 3 per cent solut on of hæmoglobin in water Ordinates = logarithm of the concentrati n f the residual hæmoglobin expressed as a percentage of the in tial concentration

(From Chick and Martin *Journal of Phys ology* 1910)

an assumption confirmed by Lepeschkin (1922) who finds that where ten seconds only is required to coagulate de natured egg white 2 220 seconds is required under similar conditions both to denature and coagulate it Denaturation therefore is a mono molecular reaction and occurs only in the presence of water The temperature coefficient of the change is high being 1 9 for 1 rise of temperature for egg

albumin between 60 and 70 C when heated in distilled water The temperature coefficient of denaturation varies with different proteins possibly also with different conditions For hæmoglobin in solution in distilled water it is 1 3 for 1 rise of temperature between 60 and 70 Curves showing the rate of denaturation over this range of temperature are reproduced in Fig 47 The linear form of the curves shows that here also the change is monomolecular

Some further evidence of the nature of the chemical change involved in denaturation is available Michaelis and Davidsohn (1911) have shown that during the denaturation of serum albumin the iso electric point changes from *p*H 4 6 to *p*H 5 4 and Chick and Martin (1911 1912 1913) that denaturation of egg albumin in acid or alkaline solutions is accompanied by combination with an additional amount of acid or base beyond that normally taken up by the unde natured protein at the *p*H of the experiment for instance a solution of 1 25 per cent egg albumin in 0 003 *N* hydro chloric acid (*p*H = 2 52) has a *p*H of 4 19 before heating and 4 71 after heating showing a fixation of acid on coagulation of 0 000038 equivalents by 10 grams of protein In 0 014 *N* hydrochloric acid the fixation of acid on heating rises to 0 00041 equivalents by 10 grams Similarly in alkaline solutions there is a fixation of base on heating These facts suggest that one change involved in denaturation may be an opening of internal salts with the release of additional amino and carboxylic groups in the molecule The action of nitrous acid on a protein before and after heating might give further evidence here Whether this change is fundamental or incidental is not clear since hæmoglobin undergoes heat coagulation in water without apparently changing its power of combining with acid

Denaturation is known in some cases to be accompanied by a change in the linkages of the sulphur atom in the molecule Raw egg white gives no colour with sodium nitro prusside in the presence of ammonia—coagulated egg white

gives a strong magenta pink showing that free sulphhydryl groups have been released during the change (Harris 1923) Serum gives no colour with sodium nitroprusside either in the raw or coagulated state but after heat coagulation it gives a magenta pink colour with sodium nitroprusside and

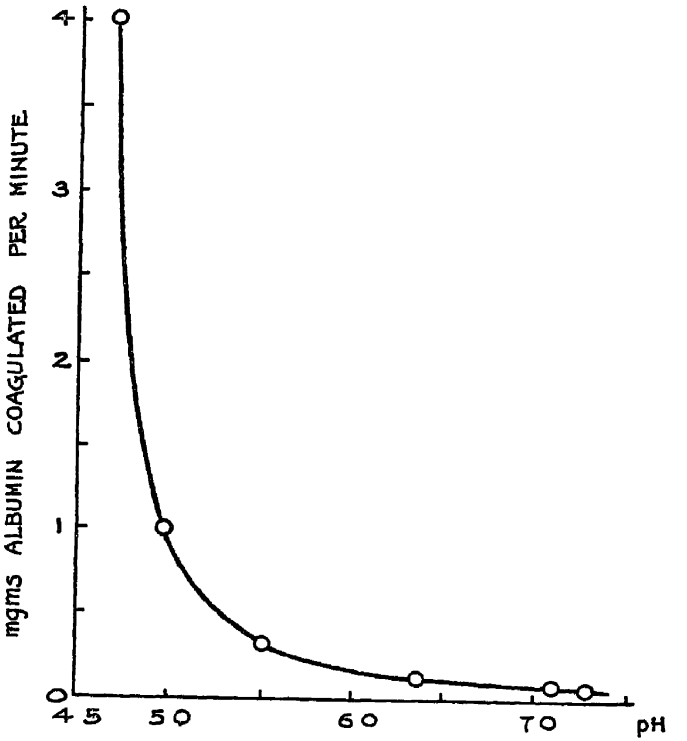

FIG. 48.—Influence of hydrogen ion concentration on rate of denaturation of crystallised egg albumin
(From Chick and Martin *Journal of Physiology* 1911)

sodium cyanide a reaction shown by Walker (1925) to be due to the development of a disulphide group

The rate at which denaturation occurs depends not only on the temperature but on a number of other factors such as the hydrogen ion concentration of the solution and the salt concentration Chick and Martin (1911) find that the minimum denaturation rate of crystalline egg albumin lies

somewhere about *p*H 7 From *p*H 7 to 5 5 the rate of denaturation at constant temperature rises slowly but with further increase of acidity it rises rapidly as will be seen in Fig 48 which shows the relation of velocity of denaturation to *p*H at a constant temperature of 69 The maximum rate shown is at *p*H 4 8 which is the iso electric point of egg albumin Chick and Martin find that *p*H 10 denaturation only proceeds slowly but at *p*H 10 4 and 11 4 the velocity rises in the latter case reaching a value nearly the same as at *p*H 4 8 Unfortunately determinations made just on the acid side of the iso electric point are not available for comparison Weber (1925) states that the muscle proteins are coagulated at high velocity particularly at their iso electric point but Homer (1917) finds that for the serum proteins denaturation is very slow between the iso electric point of the proteins and absolute neutrality but increases rapidly with increasing hydrogen or hydroxyl ion concentration outside this range 6 3 per cent of the serum protein for instance being denatured by six hours heating at 57 at *p*H 5 4 and 98 per cent at *p*H 4 6

The presence of salts *e g* sodium chloride and ammonium sulphate lowers the rate of denaturation The salt action has not been disentangled from the influence due to the alteration in *p*H caused by adding the salt but the lowering of the denaturation rate seems greater than can be accounted for by change in the hydrogen ion concentration alone Handovsky (1910) states that salt free proteins can be denatured at 35

The coagulation or separation of the denatured protein from the solution is affected by the same factors which influence denaturation though not in the same manner Lepeschkin (1922) finds for instance that the temperature coefficient of coagulation of heat denatured egg albumin by means of small quantities of ammonium sulphate is 1 2 for 1 rise of temperature over a range of from 30 to 80 He compares this with the temperature coefficient of coagulation

of arsenic trisulphide (1 05 for 1) and of lecithin (1 1 for 1) The temperature coefficient of coagulation is therefore less than that of heat denaturation at the same time as already mentioned the actual rate of coagulation is much greater than that of denaturation under the same conditions De naturation can be carried to completion in time at any reaction Coagulation on the other hand is only complete at the iso electric point of the denatured protein (Sørensen and Jurgensen 1917 Michaelis and Mostynski 1911) and takes place most rapidly in the presence of salts (Weber 1925) Chick and Martin (1912) have shown that the coagulation of egg albumin and serum is greatly affected by the reaction at which denaturation takes place If this has been in an acid solution the protein particles become positively charged if in an alkaline one negatively charged and in the absence of dissolved salts the denatured protein remains in solution Adjustment of the reaction to the iso electric point (*p*H 5 4 for denatured serum albumin) leads to the disappearance of the charge and to the precipitation of the protein At hydrogen ion concentrations removed from the iso electric point precipitation of the denatured protein by electrolytes takes place in accordance with Hardy s law (1899 1900) *i e* in acid solutions the positive protein particles are precipitated by negative ions in alkaline solutions the particles being negatively charged by positive ions In both cases the efficiency of the coagulating ion is a function of its valency Chick and Martin give a series of experimental figures which are reproduced in Table VIII

It can be seen in the acid dispersion that the agglutinating power of the negative ion is roughly

Cl SO_4 Citrate 10 10^2 10^3

Sodium citrate first agglutinates and then disperses the coagulum the particles in the latter case being negatively charged It should be noticed that the *p*H of the latter system is 6 21 *i e* the hydrogen ion concentration has gone to the alkaline side of the iso electric point

TABLE VIII

A 0·05 per cent solution of denatured dialysed egg white (*a*) dispersed by acid *p*H = 3·59 (*b*) dispersed by alkali *p*H not given

\+ = agglutination — = dispersion

Salt added	Concentration of terminal	Agglutination
	(a) *Original material dispersed by acid*	
NaCl	0·06	—
	0·08	—
	0·10	+
	0·20	+
	0·50	+
Na_2SO_4	0·004	—
	0·006	+
	0·01	+
	0·05	+
	0·5	+
Na_3 Citrate	0·0002	—
		Particles positively charged *p*H = 3·59
	0·0004	—
	0·0006	+
	0·0008	+
	0·001	+
	0·002	+
	0·004	—
	0·006	—
		Particles negatively charged *p*H = 6·2
	(b) *Original material dispersed by alkali*	
NaCl	0·01	—
	0·5	—
	1·0	—
	2·5	—
CaCl	0·01	—
	0·02	+
	0·05	+
	0·1	+
	0·2	+
	0·5	—
$La(NO_3)_3$	0·0001	—
	0·0002	+
	0·001	+
	0·01	+
	0·05	+

The lanthanum nitrate in the alkaline system is a powerful agglutinant but does not cause dispersion in higher concentrations In another experiment with dialysed horse serum denatured by heating in 0 0001 N sodium hydroxide the lanthanum nitrate brought about agglutination at a concentration of 0 00006 N and dispersion at a concentration of 0 002 N the particles in the latter case having acquired a positive charge The change in the hydrogen ion concentration was not followed in this experiment

The dispersion of the precipitated protein that takes place under the influence of the polyvalent ions accompanied by the reversal of the sign of the charge is partly to be explained by the change in reaction which occurs but that this is not the most important factor has been shown by Chick (1913) who states that euglobulin in the presence of sodium citrate can be made to take up a negative charge in solutions with a hydrogen ion concentration of $1\ 59 \times 10^{-4}$ ($p\mathrm{H} = 3\ 8$) *i e* on the acid side of the iso electric point and also that in the presence of lanthanum nitrate it can be made to take up a positive charge at a reaction of about $p\mathrm{H} = 7$ *i e* on the alkaline side of the iso electric point

To sum up the evidence on the relation of acid salts and bases to heat coagulation of proteins it may be stated that —

(1) Denaturation is due to a chemical change in the dissolved protein accelerated by a rise in temperature and by the presence of acid or alkali in the solution and retarded in acid solution by the presence of salts

(2) Coagulation or agglutination is due to the electrical discharge of the charged denatured particles It is accelerated by a rise of temperature and is only complete at the iso electric point In acid solution negative ions in dilute solution bring about agglutination trivalent ions being more effective than divalent and these than monovalent In alkaline solutions the positive ions bring about agglutination In more concentrated solutions of salts the agglutinated

protein becomes re dispersed with a change of sign Heat denatured proteins do not dissolve to form electrically neutral solutions in dilute salt solutions

Denaturation by Light

Denaturation by ultra violet light (Dreyer and Hanssen 1907) and by the rays of the visible spectrum (Young 1922 2 is a process in many ways similar to denaturation by heat and is also characteristic of the albumins and by inference of the globulins Bovie (1913) showed that the conversion of egg albumin by ultra violet light into an insoluble form took place in two stages (1) denaturation with a low temperature coefficient (2) coagulation with a higher temperature coefficient Coagulation was dependent on the presence of electrolytes The contrast between the low temperature coefficient of denaturation by light and the high coefficient for denaturation by heat is also brought out by Young

The influence of hydrogen ion concentration on denatura tion by light is also shown by Young

TABLE IX

Effect of pH *Variations on* 0 7 *per cent Egg Albumin in Buffered Solutions*

Initial *p*H	*p*H after five hours exposure to sunlight	Result after exposure	Result after exposed dialysis to *p*H 4 8
3 0	3 5	+ + + +	− + + +
4 0	4 3	+ +	+ +
4 8	5 1	+	+
5 4	5 6	Opalescent	+
6 0	5 6		+ +
7 6	6 5		+ + + +

Light denaturation therefore is accelerated on either side of the iso electric point and resembles heat denaturation

being accompanied by combination between the protein and acid or base respectively Coagulation of the denatured protein is at a maximum at the iso electric point which evidently lies between *p*H 4 8 and 5 4 The alteration in the physical state of light denatured egg albumin is shown by the fact that during exposure the viscosity rises and the surface tension falls A change in chemical condition is shown by a measurement of the optical rotation Young (1922 1) showed that the specific rotation of a protein at its iso electric point is a physical constant After denaturation there is a marked increase $[\alpha]_E$ for the mercury green line for egg albumin is — 36 6 after partial denaturation it increases to — 40 38 $[\alpha]_E$ for serum albumin is — 78 6 after partial denaturation it increases to — 89 6° With both normal and denatured proteins the specific rotatory power varies to a small extent with the hydrogen ion concentration

Denaturation by light is accelerated by the addition to the solution of substances such as alcohol acetone and certain salts (among which may be mentioned sodium chloride ammonium sulphate and potassium thiocyanate) the common property of which seems to be a capacity for binding water (Young 1922 2)

Denaturation by Strong Acids and Alkalis and the Salts of the Heavy Metals

The conversion of soluble proteins such as albumins and globulins and casein into insoluble meta proteins by the action of concentrated strong acids and alkalis has long been known The properties of the metaproteins have been given at the end of Chapter III They are closely similar to those of heat denatured proteins Some recent work on the conversion of soluble serum albumin into the de natured form is given by Wagner (1920) who finds that the acidity of the solution affects the temperature at which a visible coagulum appears This work on the effect

of concentrated acids on denaturation is supplementary to the work of Chick and Martin on dilute acids It is quite evident that both heat and hydrogen ions catalyse the chemical changes leading to denaturation If the tempera ture is sufficiently high denaturation can take place in neutral solutions if the hydrogen ion concentration is sufficiently high denaturation can take place at low temperatures (20 or even less)

The salts of the heavy metals and rare earths also cause irreversible precipitation of albumins and globulins (Mines 1911) Multivalent complex anions do not seem to have this effect Thomas and Norris (1925) found that if a solution of protein on the alkaline side of its iso electric point be taken for instance a solution in water very small quantities of a salt of a heavy metal for example thorium or zinc will serve to throw down a precipitate in which both protein and metal are present This type of reaction is used as a test for proteins (see Chapter I) Further addition of the salt leads to solution Chick and Martin showed that this re solution was accompanied by a change of sign on the protein particle and Thomas and Norris have shown that this is due to the hydrolysis of the salt with the development of an acid reac tion With further addition of salt a second precipitation occurs in the acid solution This precipitation is irreversible and is due to the formation of a denatured protein If the precipitate be washed with acid at the reaction of precipita tion it is found that the heavy metal is not combined with the denatured protein The metallic ion has acted as a catalyst for the molecular change which leads to denaturation

Denaturation by Mechanical Means

Denaturation is not only brought about by chemical reagents and temperature but has been shown to occur quantitatively under the influence of violent shaking (see Hopkins 1900) Ramsden (1903 1904) has shown that adsorption at a surface or in a film leads to denaturation

The formation of the stiff foam of egg white that results from beating the material is caused by the denaturation of the proteins which form films of solid particles round the air bubbles The factors affecting mechanical denaturation have not been studied

Egg white is also denatured by pressure at 7 000 atmospheres (Bridgman 1914) and casein a protein which is not denatured by heat is denatured by pressure on the acid side of its iso electric point (Porcher 1925)

Denaturation by Alcohol and Acetone

It has long been known that proteins precipitated from aqueous solutions by the addition to these of either alcohol or acetone are converted into a form completely insoluble in water *i e* they are denatured The relationships of the proteins thus denatured to proteins denatured by the catalytic action of hydrogen or hydroxyl ions heavy metal ions or heat has not yet been studied There is no doubt that temperature plays an important *role* in the denaturation by alcohol or acetone since Hardy and Gardiner (1910) have shown that at temperatures lower than 5 this action does not occur or only does so with extreme slowness

The Action of Lecithin

The coagulation of denatured proteins is influenced by the presence of lecithin The chemical nature of the effect is quite unknown but there is no doubt that lecithin has a strong influence on the process of denaturation There are in blood serum milk etc two forms of globulins The first euglobulin is precipitated from neutral solution by one third saturation with ammonium sulphate and by dialysis or dilution (Hardy 1905 Chick 1914) It is soluble in dilute acids and alkalis dilute salt solutions but insoluble in water The second pseudoglobulin is precipitated from neutral solution by half saturation with ammonium sulphate

it is soluble in dilute acids alkalis and water It seems possible that pseudoglobulin is the only form of globulin present in the blood of the living animal since Hardy and Gardner (1910) found that by precipitating the proteins of plasma with ice cold alcohol and subsequently dehydrating completely in the cold and extracting with ether they obtained a fine white powder completely soluble in distilled water After standing for some hours at room temperature a precipitate of globulin begins to appear due to spontaneous denaturation Euglobulin differs chemically from pseudo globulin since it contains phosphorus and Chick (1914) has put forward evidence to show that this is due to the associa tion of lecithin with the protein in an adsorption compound Euglobulin according to Chick is derived from pseudo globulin by the denaturing influence of lecithin When all the lecithin present in the serum has been exhausted by precipitation as a lecithin protein complex the conversion of pseudoglobulin into euglobulin comes to an end

Euglobulin differs from heat denatured proteins in being soluble in dilute salt solutions It can be further denatured by heat when it again becomes insoluble in these The fate of the associated lecithin under these conditions is not known

A further case where it is possible that lecithin is concerned in denaturation is given by Heilbrunn (1924) who has studied the heat coagulation of protoplasm using the eggs of *Arbacia* (sea urchin) and *Cumingia* (clam) as his material and who considers that coagulation is due to an alteration in the fatty constituents of the cell It is interesting that in this material Heilbrunn finds (as Pickering and Hewitt (1921) found for blood) that coagulation is reversible in its early stages

Theories of Denaturation

It is not at present easy to form a satisfactory theory of the cause of denaturation and it is not yet certain that the different types of denaturation *i e* by heat light mechanical means action of strong acid or alkali action of alcohol are

brought about by the same types of change in the molecule or by different mechanisms Denaturation by light or heat seem to be undoubtedly of a similar nature and the evidence concerning them may be summed up as follows This type of change seems to be confined to the albumins and globulins It leads to loss of the power of passing into solution as electri cally neutral particles to greater combining capacity for both acids and bases to an appearance of fresh reactive groups to a change in the specific optical rotation to a greater ease of attack by the proteolytic enzymes (see Chapter VI on the influence of cooking on the digestibility of egg albumin and stizolobin) and it only occurs in the presence of water Chick and Martin suggest that denaturation consists of an opening up of internal anhydride rings with combination with the elements of water at the point of cleavage This theory would account for all the known facts on denaturation with the exception of the loss of solubility anhydrides being in general less soluble than the bodies from which they are derived Young suggests that denaturation consists of the closing of rings and formation of internal anhydrides This theory would explain the loss of solubility in water and dilute salt solutions but leaves the appearance of the sulph hydryl group in denatured egg albumin of the disulphide group in denatured serum albumin and the greater digesti bility by the proteolytic enzymes entirely unexplained

Wu and Wu (1925) believe that denaturation by heat is a hydrolysis resulting in an actual cleavage of the molecule basing their opinion on the evidence that after heating in dilute solutions of acid there is an increase in the concen tration in the solution of substances which give the colour reaction for tyrosine but which are not thrown out of solu tion by protein precipitants

Of the other types of denaturation even less is understood than in the case of heat and light The action of strong acids and alkalis and especially of alcohol suggest that the change in these cases is more likely to be due to condensation and

dehydration than to an opening of anhydride rings but the very scanty information available suggests that judgment should for the present be reserved

Denatured Proteins in Physiology and Industry

The study of denatured proteins has many applications both in physiology and industry The tissue of the body

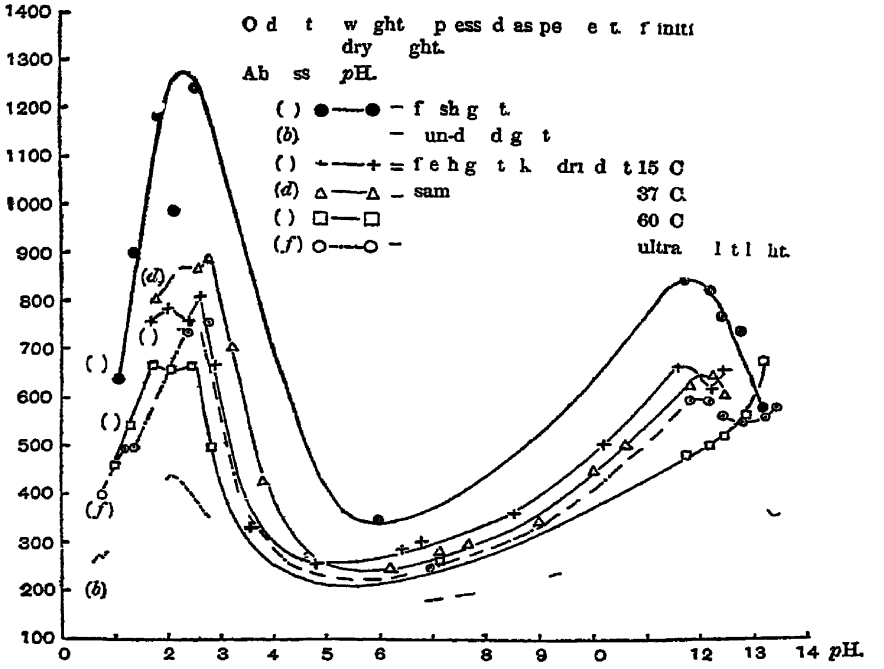

FIG 49 —Curves showing the reduction of the water absorbing power of goat skin brought about by drying the skin under conditions which favour the denaturation of the interfibrillary proteins
(From Kaye and Jordan Lloyd *Biochemical Journal* 1925)

which probably receives most light is the lens of the eye which consists very largely of two globulins α and β crystallin The clouding of the lens which occurs in old age cataract is probably due to the slow denaturation of the lens proteins under the action of light (see Schanz 1915 1916) The condition known as glass workers cataract has also been

shown to be due to damaging action of the infra red rays irradiating out from the molten glass It is well known that cataract is a condition incurable in the sense that the original transparency of the lens cannot be restored and it is therefore probable that it is associated with an irreversible alteration in the lens proteins

In industrial conditions denaturation is generally a change

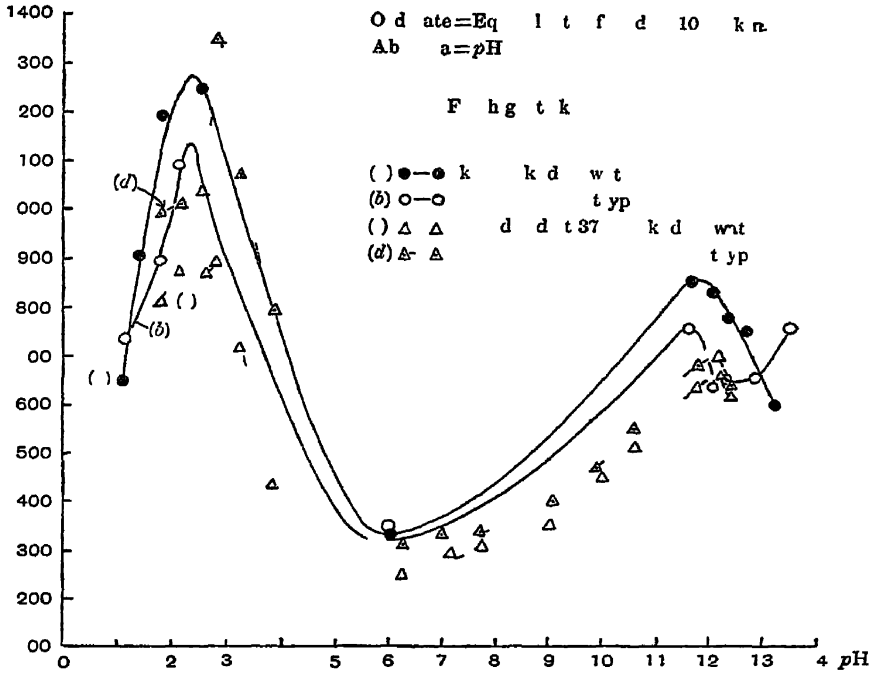

FIG 50 —Curves showing the restoration of the swelling power of goat skin by treatment which removes the coagulated interfibrillary proteins (From Kaye and Jordan Lloyd *Biochemical Journal* 1925)

which it is desirable to avoid In the making of milk powders the drying of the milk by evaporation at a high temperature leads to a coagulation of the lactalbumin and lactoglobulin unless it is carried out at great speed The coagulation of these proteins results in a preparation containing an insoluble residue and as already mentioned in Chapter VI the coagulable proteins of milk are a very

important part of the milk from a dietetic point of view The method of manufacture of dried milk has been briefly described in Chapter VI Similarly in preparing dried eggs it is important to avoid denaturation of the proteins

Another condition under which coagulation may occur is in the drying of animal skins for export as a raw material for leather manufacture Kaye and Jordan Lloyd (1924) have shown that the proteins of the interfibrillary lymph can be coagulated during drying both by heat and light and that the result of coagulation is by forming an insoluble non elastic layer round the fibres to check the absorption of water which is a necessary preliminary to the use of the skin for the manufacture of leather The sheath of coagulated protein forms an additional mechanical hindrance to the swelling of the fibres It can be seen from Fig 49 how drying at 60 or under a strong source of ultra violet light reduces the subsequent power of swelling The dispersal of the sheath of coagulated protein round the fibres by the action of sodium citrate or its removal by digestion with trypsin restores mechanical freedom to the fibres so that they can again absorb water and swell in the proportions characteristic of fresh skin (Fig 50)

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CHAPTER XIV

IRREVERSIBLE PROTEIN GELS

The Clotting of Blood—The Clotting of Milk and the Chemistry of Cheese making—The Metaproteins

The Clotting of Blood

Under certain conditions enzyme action can lead to the irreversible precipitation of dissolved proteins by a process very similar to denaturation The precipitates frequently take the form of irreversible gels The heat reversible gel of gelatin is an isolated phenomenon peculiar to this one protein Its chemical basis is a reversible change between two chemically different forms of one substance a change controlled by the temperature The production of an insoluble gel forming condition of certain other proteins as a result of enzyme action is however an irreversible change best designated by the term clotting of which the two best known examples are the clotting of blood and milk

The factors controlling the spontaneous clotting of blood have been the subject of a considerable amount of research and the mechanism by which the change is brought about is yet not fully understood The following facts however seem to be fully established Blood plasma (*i e* blood freed from corpuscles) is a solution containing three proteins possibly associated into a loose complex These are fibrinogen serum albumin and serum globulin The power of forming the clot is confined to the fibrinogen Besides the proteins in the plasma there are also present inorganic metallic ions sodium potassium calcium etc and an inactive enzyme

pro thrombin When blood is shed a co enzyme thrombo kinase is released by the white corpuscles or leucocytes of the blood and the injured tissues It is interesting to notice that here as in the autolysis of tissues the enzyme system which is always present in the living system only becomes active in the presence of injured or dying cells This enzyme system thrombin (*i e* pro thrombin + thrombokinase) acts on the fibrinogen of the plasma transforming it into a product which is precipitated from solution by the calcium salts present and forms the elastic clot Barratt (1920) has shown that the fibrinogen comes out of solution as long fibrils These slowly contract on standing squeezing out water in which albumin and globulin are still present in solution This expressed fluid is the blood serum The three factors fibrinogen calcium and thrombin have all to be present to ensure spontaneous clotting Blood shed into a solution of sodium oxalate loses its soluble calcium ions and will not clot spontaneously Sodium fluoride in some manner not understood inhibits the production of throm bokinase and blood shed into sodium fluoride does not clot The inter action between the three factors is not yet fully understood but it seems probable that the enzyme thrombin acts on the fibrinogen causing some structural change in its molecule and that the *denatured* fibrinogen or fibrin is then precipitated from the slightly alkaline solution by the calcium ions present Pickering and Hewitt (1921) consider that the first stage in clot formation is the destruction of an association between fibrin and a protective colloid In support of this hypothesis they have shown that in its earliest stages the clot formation can be reversed In its later stages however the change is irrever sible and must therefore be accompanied by some chemical change in the molecule It is obvious how closely parallel the change is to the denaturation by heat which is charac teristic of all the animal globulins Hirsch (1924) has shown that the hydrogen ion concentration of the blood changes

during clotting becoming more acid from which it follows that as in heat denaturation some of the base present has combined with the denatured protein in this case fibrinogen Fibrinogen can be induced to clot by a number of other agencies among which serpent s venom may be specially mentioned (Barratt 1920)

The Clotting of Milk and the Chemistry of Cheese making

Another example of clot formation by enzymes occurs in cheese making Milk is a solution containing three proteins caseinogen lactalbumin and lactoglobulin also probably associated into a loose complex and inorganic metallic ions among which is calcium Cheese is made from milk by the action of rennin an enzyme derived from the walls of the stomach of the calf but not to be confused with pepsin The evidence of the individuality of rennin and pepsin has been clearly summarised by Cole (1919) The rennin acts on the caseinogen converting it into casein which is precipitated from alkaline solution by the calcium ions and forms a clot which shrinks gradually expressing the milk serum or whey in which the albumin and globulin remain in solution It can be seen that the clotting of blood and the clotting of milk to make cheese are so far closely similar processes Alexander (1910) con siders that in milk casein is present associated with lactal bumin which acts as a protective colloid and that it is to the removal of the latter from the association that the clotting is primarily due The later stages of cheese making known as ripening are not due to further physico chemical changes in the casein but to chemical disintegration brought about by micro organisms which hydrolyse a proportion of the proteins in the cheese release free amino acids and then oxidise these for use as food leaving in the cheese end pro ducts which give it the required characteristic flavour It is important in cheese making to have the right micro organisms (bacteria and moulds) growing in the cheese and to ensure

successful work in any new factory it has long been the practice to rub over the shelves with the rind of a cheese brought from an old factory This ritual suggestive at first sight of sympathetic magic is now recognised to have a sound scientific justification It will be pointed out in Chapter VII that even with the right species of bacteria the end products produced are determined largely by the chemical nature of the growth medium and the physical conditions and it follows therefore that successful cheese making requires a careful control not only of the composition of the milk but also of the physical conditions accompanying the processes of clotting and ripening

The type of cheeses made by the action of rennin are known technically as hard cheeses They consist of such forms as Cheddar Cheshire Gruyere Edam etc Their characteristic feature is the very great degree of shrinkage that takes place during manufacture with a corresponding loss of water There is however another class of cheese known as soft cheeses which are principally milk and cream cheeses In these cheeses clotting is brought about by acid and pressure and is a typical case of denaturation There are no stages of ripening and soft cheeses therefore have all a very similar taste The clot of soft cheeses does not shrink to any great extent and they therefore hold more water and are softer in consistency than hard cheeses In making soft cheeses the milk or cream is allowed to go sour *i e* acid forming bacteria are allowed to develop These bring the reaction of the milk from a *p*H of about 7 to 8 to a *p*H of 4 to 5 with a consequent precipitation of the casein as the reaction approaches the iso electric point In hard cheeses the calcium present is largely precipitated with the clot in soft cheeses it is mainly left in the whey Although slightly sour milk can be saved from precipitation by the addition of traces of alkali once the clot is formed it is impossible to reverse the condition

Lindet (1925) considers that the coagulation of the clot

cannot take place in acid solution unless calcium ions are present and Porcher (1925) working with him finds that the coagulum formed by compressing the acid casein is correspondingly firm in proportion to the amount of calcium phosphate present Neither Lindet nor Porcher consider however that the calcium ions are in any way combined with the casein of the clot

The Metaproteins

Not only rennin and thrombin but also preparations of the hydrolytic enzymes seem to act at first on a protein substrate by converting it into a metaprotein the properties of which are in many ways akin to those of proteins denatured by chemical and physical means Wasteneys and Borsook (1924) define metaprotein as a condition in which the protein is not precipitated by trichloracetic acid but is precipitated or coagulated by adjustment of the reaction to a definite value of *p*H In many cases of enzyme hydrolysis the first stage of the reaction is the precipitation of the metaprotein in the form of a gel but it is not yet clear whether this is brought about by the hydrolytic enzymes themselves or by bodies of a rennin like nature associated with them

The structural relation of the proteins and the meta proteins is not yet clear nor is that between the meta proteins produced by enzyme action and those produced by the action of strong acids It has been suggested that the change in both cases is due to the splitting of internal anhydride rings but it is difficult to see why such a change should reduce the solubility at the iso electric point and at the same time preserve the molecule from the precipitating action of such reagents as phosphotungstic acid trichloracetic acid colloidal iron etc It is safer to admit for the present that the conversion of a protein into a metaprotein is due to an undiagnosed chemical change It is not necessary to assume that because the change is the first stage in digestion it is therefore due to the hydrolysis of a peptide link with a

splitting of the molecule or even to the opening of a diketo piperazine ring Many proteins are made more susceptible to the action of the hydrolytic enzymes by previous cooking (denaturation by heat) and it is quite conceivable that in the stomach there is an enzyme (rennin) the function of which is to denature the food proteins or in other words to catalyse a reorganisation of their molecules that will leave them in a condition in which they will be susceptible to the action of the hydrolytic enzymes

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CHAPTER XV

THE INDUSTRIAL USES OF THE PROTEINS

The Uses of the Proteins—The Keratins and Textile Manufacture—Collagen and Leather Manufacture—Adhesives—Glazes Varnishes Distempers and Sizes—Galalyte—Protein Sols and Gels in Industry

The Uses of the Proteins

The most important economic function of the proteins is as food for man and his domestic animals In this domain they cannot be replaced by any other class of substances and the turning of food proteins to industrial purposes would in general be economically unsound Only proteins which have no food value (the keratins collagen and gelatin) or which can be produced abundantly and cheaply (casein and gluten) are suitable as raw materials for industry Attention has already been drawn to the fact (see Chapter I) that nearly all the important industrial proteins are of animal origin The animal uses protein for building up tissues or other structures with specialised functions and in many cases it is the histological structure of these extra cellular proteins which gives them their industrial importance For instance it is the naturally occurring thread like flexible form of hair wool and silk among the keratins that gives them their importance in the textile industry and it is the closely woven fibrous structure giving both flexibility and toughness that has made the skin the basis of leather manufacture If skin fibres had originally been evolved from carbohydrate instead of collagen skin would still have gone to make leather but the chemistry of tanning would have been different The industrial proteins however are not limited to those with a special histological structure Casein

gelatin and other structureless proteins have an important place in industry on account of the colloidal properties of their solutions The protective or stabilising properties are important in pharmacy and the preparation of photographic gelatins the surface adsorption of suspended particles in the fining of beer the power of colloids to inhibit crystallisation in the confectionery trade and the tendency of the particles to adhere on drying and form continuous films is the basis not only of the adhesives industry but also of the use of proteins in distempers glazes and dressings for textiles and paper The important industrial properties of protein solutions is in every case due to their colloidal state In many cases they are used as an alternative to the colloidal carbohydrates The chemical properties of the proteins are only of indirect importance in industry

The Keratins and Textile Manufacture

The main groups of the protein industries may now be considered separately If for convenience the order of review passes from insoluble to soluble proteins the first class to be considered is the keratins These exist in nature as firm insoluble bodies which are immune from bacterial attack Some of them such as tortoiseshell and horn can be used directly for the manufacture of buttons combs and other small articles of commerce Others such as hair wool and silk are formed as long pliable fibres which are ideal for weaving into textiles and their use for this purpose is as old as history The keratins need little or no chemical treatment to fit them for industrial use but by virtue of the amphoteric character which they possess in common with all other proteins the final article can readily be dyed by either acidic or basic dyes Modern chemistry has taught the textile manufacturer how to control the colour and texture of his silk or woollen fabric but the essential chemical and physical condition of the keratin fibre is not changed either in the weaving or the finishing process

Collagen and Leather Manufacture

In the great industry of leather manufacture the object to be attained is the maintenance of the histological structure with the transformation of the protein basis into a more stable form The raw material for this industry is animal skin a tissue in which the close interweaving of numerous fibres during life freely moveable over one another results in a combination of toughness and flexibility which has no counterpart either among natural products or industrial artefacts Unfortunately however after flaying the skin starts to lose water by evaporation and the collagen of which it is composed develops the adhesive qualities characteristic of all drying proteins with the result that the fibres now stick together and so flexibility is lost If the water of the flayed skin is not allowed to evaporate bacterial putrefaction sets in and moreover both fresh and dry skins have swelling properties that would be distinctly inconvenient in the sole of a shoe or a strap round a parcel ! In turning putrescible skin into non putrescible leather water must be eliminated but the freedom of the fibres must be maintained and the protein changed to an insoluble and non absorbent form The chemistry of leather making is the application to industry of the chemistry of the insoluble protein derivatives

Vegetable Tanning—It will be recalled that a general property of all proteins is that they are precipitated by tannic acid from weakly acid solution and the most general method of converting skin into leather is by impregnating the skin with a water extract containing one or more of the vegetable tannins or tannic acids Skins are prepared for tanning by steeping in saturated lime water This process removes the hair and even after deliming leaves the skin in a porous condition favourable for the penetration by the tannins which are colloidal in solution the particles carrying a negative charge (see Procter 1922) As a result of tanning the collagen fibres of the skin become impregnated

with the tannin lose their water and their power of swelling and can be dried without adhering The chemistry of vegetable tanning is not quite clear Proctor and Wilson (1916) put forward the theory that in the weakly acid reaction of the tan liquids the collagen functions as a base and forms ionisable salts with positively charged colloidal ions which are precipitated by the negatively charged colloidal ions of the tannin solution The iso electric point of collagen is at *p*H 4 8 according to Porter (1921) *p*H 4 6 according to Wilson and Gallun (1923) and *p*H 5 0 according to Meunier Chambard and Jamet (1925) On the colloidal precipitation theory tanning should only occur in reactions more acid than *p*H 4 6 Thomas and Kelly (1923) have shown that although the fixation of tan by skin is at a minimum at *p*H 5 0 and rises to a maximum between *p*H 2 0 and 3 0 there is also a secondary maximum between *p*H 7 0 and 8 0 and that fixation of tan extends beyond both these maxima The mutual precipitation of oppositely charged colloids undoubtedly plays an important part in the combination of tan and hide fibre but does not form the complete story Some chemical interaction between tannin and protein seems also to occur

Mineral Tanning —If precipitation of an insoluble collagen compound is the essential process in tanning then other protein precipitants should also be able to function as tanning agents Under certain conditions salts of certain metals such as iron chromium etc can precipitate proteins from their solutions and iron chromium manganese aluminium have all been shown capable of converting skin into leather Alum tanning is an ancient form of the tanner s art but chrome tanning is the mineral tannage at present of chief commercial importance Alum tanning or tawing can be reversed by extraction with water but the collagen chromium complex is stable even in the presence of boiling water and the chrome tanned fibres do not swell in acid or alkaline solutions The chemical reaction that takes place between the chromium and

the collagen is not at present understood Basic chromium sulphate is very generally used for tanning but the tanning solutions are always acid showing that the chromium ion as such (positive) will not be interacting with the positive collagen ions It seems more likely that the collagen reacts with a negative chromium complex and that there is probably a deposition of an insoluble chromium salt acting as lubri cating layer round the fibres Kubelka (1923) has shown that hide powder takes up more chlorine ion than metallic ion from solutions of either aluminium or ferric chloride thus leaving a more basic salt behind in the solution

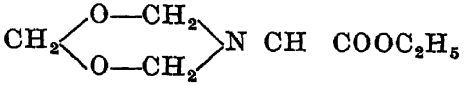
Silica which precipitates gelatin under certain conditions has also been used as a tan

Formaldehyde—Another reagent which reacts with pro teins turning them slowly into a form which is insoluble even in boiling water and does not swell in acid or alkali is formal dehyde This substance can also be used as a tan The reaction between formaldehyde and the amino groups of the amino acids is well known and is the basis of Sørensen s method of estimating the latter in solution It is usually expressed as

$$\begin{array}{l} R\text{——}NH_2 \\ | \\ COOH \end{array} + H\ CHO \longrightarrow \begin{array}{l} R\text{——}N\ \ CH \\ | \\ COOH \end{array} + H_2O$$

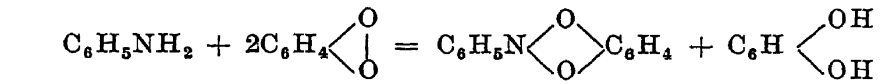
(see however Harris 1925) Formaldehyde also combines with proteins including gelatin and collagen Stiasny (1908) observed that formaldehyde treatment of skin reduces its swelling power and its capacity to combine with acid Gerngross and Loewe (1922) recorded that formaldehyde treatment increases the amount of base which a skin can absorb Bergmann (1923) considers that formaldehyde can combine not only with amino groups but also with the pep tide group diketopiperazine for instance combining with formaldehyde in the molecular ratio of 1 2 Bergmann finds that glycocoll acetic ester forms a stable compound

with formaldehyde in a molecular ratio of 1 3 He gives as the formula



Formaldehyde leather is therefore produced by the formation of insoluble chemical compounds between the collagen and the formaldehyde The reactions are reversed in the presence of decinormal hydrochloric acid or even slowly on long standing in moist air Nevertheless the reaction between proteins and formaldehyde has constantly been used in industry for producing an insoluble derivative of a protein

Quinone and the Halogens—Meunier (1908) showed that phenols nitrophenols and chlorophenols all precipitate gelatin from solution In neutral or acid solutions the precipitate can be redissolved in alkaline solutions on standing in contact with air it slowly becomes transformed into a form insoluble in acids alkalis or boiling water and which no longer has the power of swelling Meunier compared the action of quinone on proteins to its action on aniline



The reaction involves an oxidation of the aniline with a simultaneous reduction of part of the quinone to hydro quinone Meunier has shown that quinone tans skin to a very resistant white leather with the simultaneous production of hydroquinone (1908 2) He believes that oxidation of the collagen produces an irreversible change in the protein and that many oxidising agents might therefore be used as tans He found that chlorine and bromine precipitate gelatin from solution and that the precipitates slowly become insoluble in water acids or alkalis Iodine does not have this effect Meunier (1911) showed that chlorine and bromine can both act as tanning agents and considered that they function through their oxidising action on the protein

Oil Tannages—It is interesting to notice in connection with Meunier's theories that chamois leather so well known for its resistance to damage by water is manufactured by impregnating skins with oil and that the process is accompanied by a great development of heat which has long been considered to be due to oxidation

It is unlikely that one type of chemical change only underlies the various methods of tanning It is interesting however that the amino group of the protein has become involved in every theory of tanning from colloidal precipitation to oxidation Rakusin (1922) points out that in addition the peptide link seems to be involved since he finds that after tanning skins with vegetable tannins formaldehyde or phenols the resulting leather no longer gives the biuret reaction The various theories and methods of tanning are described in detail in the text books of Procter (1922) and Wilson (1923)

Histological Fixatives—The conversion of the cell proteins into permanently insoluble stable forms is the object of the fixing reagents with which fresh tissues are impregnated previous to sectioning for microscopical examination The fixing process is therefore essentially a tanning process applied to albumins and globulins as well as collagen The common fixing reagents formaldehyde picric acid chromic acid etc all have tanning properties The special technique of fixing is due to the fact that the tans have to do their work throughout a cellular as distinguished from a fibrous tissue

Adhesives

The object of the leather manufacturer is to eliminate the water from skin making it durable and non putrescible while at the same time preserving the qualities due to its histological structure Tanning is a process designed largely to overcome the natural cohesive tendencies of the drying collagen fibre In the adhesive and allied

industries these same properties of the colloidal protein particles are the basis of the industry Drying proteins not only cling to themselves but also to a greater or less degree to many other substances with which they may be brought in contact In the adhesives industries the proteins used do not as in leather manufacture have to undergo a chemical change in order to be made suitable for the purpose of the industry The chemistry of the protein adhesives is the chemistry of preparing a pure soluble form of the protein required Nevertheless adhesive properties are not a special property of the proteins as a class they are also a property of the complex carbohydrates the starches and the mucilages or gums Cohesion and adhesion are nowadays attributed to residual fields of force which are the result of the large size and complicated structure of the molecule The strong attractions between the molecules prevents proteins on drying from crumbling to powder They dry to hard horny masses from which it is very difficult to remove the last traces of water

For use as an adhesive the protein must be in a form soluble in water Albumin which is freely soluble in water is occasionally used but the two standard adhesives are gelatin which is transformed into a soluble form by heating and casein which insoluble itself dissolves readily in the form of its calcium salts Gelatin is derived from collagen and is manufactured from skins or bones by a gentle hydrolysis with dilute acid under the action of high pressure steam Unless the process is very carefully controlled hydrolysis not only of the collagen but also of the gelatin will occur The properties of the resulting gelatin or glue are affected by the degree of degradation Bogue (1920) finds that the adhesive properties of a good gelatin are increased up to 5 per cent hydrolysis but beyond this they fall steadily The standardisation of gelatins and glues for commercial purposes is often based on a study of the properties of their solutions The most useful

test for grading glues since it can be performed rapidly is a viscosity measurement This is carried out in a 15 per cent concentration at a temperature of 35 by some standard type of viscometer Bogue (1920 1923) states that 75 per cent of commercial gelatins and glues can be satisfactorily graded on the viscosity measurement Wislicenus and Lorentz (1924) find that gelatins and glues can be satisfactorily graded by determining the percentage that is adsorbed from aqueous solution by fibrous alumina (*fasertonerde*) This material adsorbs the intact gelatin molecules but leaves all stages of degraded protein in solution The adherent properties of molecules whether protein or carbohydrate are undoubtedly influenced by the size of the molecule With diminishing size the attraction between molecule and molecule also diminishes Peptones have no adhesive properties Since viscosity is an indication of the volume of a solution which is occupied by the dissolved substance and since protein molecules are associated in solution with large quantities of water thereby occupying a relatively greater volume than the peptones which are derived from them it is easy to see why adhesive properties and viscosity should be so closely allied Another test for grading gelatins and glues is the jelly strength This is crudely performed by casting 1 inch cubes of 5 per cent concentration allowing them to set and testing the elasticity by pressing with the finger This test is also a rough measure of the proportion of undegraded gelatin in the gel The estimation of the water soluble nitrogen in a gelatin is a measure of its content in hydrolytic products The adhesive properties of gelatin belong to the intact protein (Bogue 1920 2) The higher the viscosity and the jelly strength and the lower the water soluble nitrogen the greater will be the adhesive power A chemically pure gelatin however cannot form stable gels and loses its adhesive power The concentration of intact protein in a gelatin affects its adhesive properties and the physical condition is also an important factor The hydrogen ion

activity of the dissolved protein influences adhesive properties viscosity and jelly strength The co relation between this factor and the three qualities of the solution is not however a simple one (see Sheppard and co workers 1920 1921 1922) A full account of the chemistry and technology of the gelatin and glue industry has been written by Bogue (1923)

Casein also can readily be obtained in large quantities and exhibits the same adhesive properties on drying from a solution in water Casein has the advantage over gelatin that it is prepared by precipitation from a fluid (milk) not by extraction from a tissue It is therefore far less liable to hydrolysis during the preparation The casein molecule however undergoes some change during drying which affects its solubility Freshly precipitated casein is soluble on either side of its iso electric point (*p*H 4 6) dried casein is insoluble in water and acids and frequently only partially soluble in alkalis This partial solubility affects its adhesive properties for small granules of swollen but undissolved casein will make weak places in an adhesive film The industrial uses of casein are dealt with by Scherrer (1921)

Glazes Varnishes Distempers and Sizes

The adhesive properties of gelatin and casein are made use of in other directions besides that of glueing two surfaces together The property of both proteins of forming a continuous film which shrinks during drying is made use of in giving continuous smooth surfaces to other materials Paper cardboard and even wood are frequently glazed by a thin coating of gelatin The glaze can afterwards be water proofed by means of a weak formaldehyde solution In the case of banknotes the paper is sized with gelatin which is then hardened with alum Cold water distempers are made by mixing casein lime and some pigment The presence of the lime causes the casein to dissolve as

calcium salts and the colloidal solution obtained keeps the pigment evenly distributed On drying the casein helps to form a continuous smooth surface which adheres closely to the wall If the distemper is treated with formaldehyde the casein becomes converted into an insoluble form and the surface is now washable Size which is painted on to wallpapers to make a non porous surface which can afterwards be varnished is only a low grade glue Leather is glazed on the surface by means of blood white of egg isinglass These give a continuous smooth surface which is afterwards polished with a glass roller The heat of polishing makes the glaze insoluble In the textile industry woven materials are often finished with a dressing to give them a good surface This is occasionally made from gelatin more generally from a paste of wheat flour used because of the cohesive properties of the gluten

Galalyte

The property of drying in an adherent condition does not depend on the protein being in a film Both gelatin and casein but especially the latter have been used in the preparation of compositions which can be pressed and dried in moulds for the manufacture of small articles such as buttons knobs handles combs etc The casein preparations contain lime and are always made insoluble by the action of formaldehyde So far it has not been possible to make articles of any great thickness since there is considerable shrinkage on drying which results in deformity and a liability to crack Most of the casein compositions are cloudy but galalyte the preparation of which is described in Scherrer s book (1921) is transparent It is very hard and is used for making artificial amber

Protein Sols and Gels in Industry

The colloidal properties of proteins in solution also have their uses in industry The proteins are emulsoid colloids

and as such have the power of stabilising suspensions of suspensoid colloids and of acting as emulsifying agents between two non miscible fluids This colloidal protective action is made use of in pharmacy to prepare colloidal silver Collarsol silver used externally as an anti septic and in certain cases internally as a remedy for septicæmia is a colloidal suspension of silver in water stabilised by gelatin Gelatin is also used in pharmacy as an emulsifying agent (see Holmes and Child 1920) Egg white is sometimes used to protect suspensions and stabilise emulsions Proteins not only stabilise emulsions of two liquids but also foams or emulsions of gases in liquids gelatin for instance is used to stiffen artificial whipped cream and casein is added to shaving soap to stiffen the lather Emulsed colloids not only stabilise colloidal suspen sions but they inhibit the formation and growth of crystals in saturated solutions In the confectionery trade gelatin is sometimes added to syrups to prevent the sugar crystal lising out on cooling as in marsh mallows for instance The same principle lies at the basis of the methods of manufac ture of photographic gelatins sodium bromide and silver nitrate are mixed in the presence of gelatin and the photo sensitive silver bromide is precipitated in the form of a very fine grained suspension which dries evenly on the plate or film The very fine Lippmann grainless emulsions are produced by adding gelatin to the silver nitrate and potassium bromide solutions separately before mixing This is the method of double protection (see Alexander 1923) A therapeutic application of the same principle is the addition of gelatin to cows milk when given to delicate babies since by this means the casein is prevented from forming large coarse curds (Alexander 1910)

The surface attractions of gelatin particles in solution can be used not only to stabilise but also to remove large particles causing turbidity Beer is fined or cleared by means of solutions of isinglass or gelatin A strong hot

solution is poured into the beer and settles down as a gel The tannin present helps coagulation and the turbid particles are carried down in the coagulum The well known method of clearing soups and jellies by egg white is also based on the carrying down of particles by surface adsorption on a protein coagulum the coagulation of the egg white being induced by heat Extracts of the vegetable tannins are freed from undesirable colouring matters by the addition of blood or albumen which adsorbs the pigment and can be removed by heat coagulation

The examples given in this chapter do not exhaust the uses of the proteins in industry but the main lines of application have been indicated Industrial proteins are either important on account of their natural histological structure (hair wool silk leather) or on account of the colloidal properties of their solutions In the latter field the colloidal carbohydrates can often be used instead The great advantage of many of the proteins for use in manufacturing processes lies in the fact that they can be obtained in solution for any desired manipulative process (such as glazing moulding etc) and that after drying and when all manipulative processes are over they can be fixed or made insoluble by simple chemical treatment In this important property the chemical properties of the proteins give them a particular value

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